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THE UNIVERSITY OF ALBERTA

IMMUNE RESPONSE OF THE GNOTOBIOTIC CHICKEN

by

© ELEANOR BERGEN MACKIE

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA
FALL, 1975

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "IMMUNE RESPONSE OF THE GNOTOBIOTIC CHICKEN" submitted by Eleanor Bergen Mackie in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

The purpose of this work was to examine the effect of the gnotobiotic environment on the immune competence of the chicken, particularly as measured by the graft-versus-host reaction (GVHR). Two assay methods, the chorioallantoic membrane pock (CAMP) and weight of embryo spleen (MES) were used. Genetically selected chickens, differing at the major histoincompatibility locus (B) were used as GVHR donors, with or without prior treatment. The gnotobiotic chickens did not attain the level of GVHR competence of the conventional chickens. Neonatal thymectomy of the donors lowered the immune competence of gnotobiotic and conventional chickens, as measured by the GVHR to major histoincompatibility antigens. Thymectomy stimulated immune competence of conventional chickens as measured by MES-GVHR to minor histoincompatibility antigens. Thymectomy did not stimulate the immune competence of gnotobiotic chickens tested in the same way. This confirms and explains an earlier report. Bursectomy had no effect on GVHR competence. Earlier reports of a B-associated difference in immune competence were confirmed in the gnotobiotic environment. This proves that the B-associated difference is not a secondary effect of differential susceptibility to infection.

The acquisition of "natural" antibodies to human blood groups of the ABH(O) system was slight in the gnotobiotic environment. Conventionally housed animals on a conventional diet all acquired a low antibody titre detectable with human A_1 cells, a moderate titre detectable with B cells and a greater titre of antibody detectable with human O cells. This antibody decreased at about four months of age and increased again thereafter indicating a fluctuation of unknown cause despite continuous natural stimulation. Evidence is presented that the presence of the $\underline{B}^{14}$ allele promotes the acquisition of antibody against human blood groups. Thymectomy decreased and bursectomy obliterated the acquisition of "natural" antibodies.

Data are presented in support of the following. The development of natural cellular immunity, as expressed in the GVHR, and the development of the humoral response, as expressed by the level of natural antibodies, progress independently. The spleens of female embryos used as recipients in the MES assay weighed more than male embryo spleens. Infectious delayed-type hypersensitivity is developed in the conventional, microbial environment only. The cellular immune mechanisms responsible for infectious delayed-type hypersensitivity show no mutual interaction with those responsible for GVHR. Surgical bursectomy decreased drastically the primary antibody response to sheep red cells in both gnotobiotic and conventionally housed animals. The effectiveness of chemical bursectomy using cyclophosphamide could not be established in either environment. In the gnotobiotic environment spleen weight was directly proportional to body weight of the chicken whereas this did not hold true with conventionally housed chickens. The general well-being of gnotobiotic animals was superior to the conventionally housed.

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INTRODUCTION

Germfree animals free of all demonstrable bacteria, fungi, ecto- and endoparasites, as well as gnotobiotic or "defined flora" animals can serve as a useful tool in the investigation of a variety of biomedical problems by virtue of their freedom from invading bacteria or through a specific knowledge of the nature of the organisms inhabiting these animals. Miyakawa _et al_. (1971) reviewed the utilization of the germfree animal in various situations including the study of inflamation and the etiology of infectious diseases of both viral and bacterial origin. Viral infections of germfree animals were reviewed in detail by Grieseman (1968). In animals which were neither diseased nor immune a uniform response to infection was observed indicating that the variable response of conventionally housed animals can be due to host variations rather than a difference in the virulence of viral preparations.

Most of the germfree and gnotobiotic work has been with mammals. Although larger animals such as the lamb, swine, dogs and cats have been used with reference to particular infectious diseases and protective immunity to them, most immunological work has been done on germfree rodents. In spite of the fact that the chicken was the first germfree animal developed (Reyniers _et al_., 1949) very little immunological work has been done with gnotobiotic chickens. Reyniers tested for "natural" antibodies in one Wyandotte cock, one Wyandotte hen, and one of their offspring, to red blood cells of various species including man (but not specifically for any particular human blood group) and several bacteria. Thorbecke et al., 1957, examined the lymphoid tissues of germfree and conventional chickens histologically and determined the gammaglobulin content of the serum. The differences in gammaglobulin were negligible up to the eighth week of life at which time the germfree reached a plateau while the conventionally housed showed an increase, more pronounced after ten weeks of age. Wostmann and Olson, 1964, measured precipitating antibody to bovine serum albumin and found that the germfree chicken required one more day to produce detectable antibody and produced one-half the amount of antibody at peak production, but maintained a proportionally higher level of antibody after peak production. Virtually no work has been done on the cellular aspects of immunity.

Contributing factors for this lack of work with the species lies specifically in the laborious care (Wagner, M., 1974, and Coates, M.E., 1974) involved in the husbandry of the adult animals in isolation facilities. The higher body temperature of the avian species brings about water condensation where isolators are not in rooms with constant air flow increasing the possibility of wetting filters with resultant contamination. Preventing feathers, dander, scattered feed, etc. from penetrating ports, exhaust sumps or filters with subsequent plugs or leaks requires constant vigilance and frequent transfer to new isolation facilities.

The large size of the animals requires frequent introductions of sterile feed and water with the accompanying risk of contamination. The labor and precautions required by each sterile introduction of food and water demand daily attention which rapidly becomes the predominant use of one person's time. A result of these factors is the increased frequency with which the isolation facilities must be tested or monitored for contamination. Many and various manuals for the isolation and identification of microorganisms are available and the choice of regimen and types of media for screening and monitoring is largely a question of judgment, but the information obtained from a given choice depends on an appropriate knowledge of microbial taxonomy, habitat and ecology, as well as the mechanisms of pathogenicity, growth and cultural requirements, and a knowledge of general diagnostic procedures and techniques. The choice of methods and the reasons for these choices are given in Materials and Methods. At the present time, other workers employing the species tend to confine their experiments to an early age such as six weeks in the study of synergism of microorganisms of the respiratory tract of gnotobiotic chickens (Springer, W.T. et al., 1974) and about four months in nutritional studies (Coates, M.E., 1974). This study employs animals up to the age of six months (albeit gnotobiotic and not germfree). One group was maintained totally germfree for a period of eight and one-half months which included four months of the first laying period.

Insofar as the prenatal animal is germfree, the nonmicrobial status is not "abnormal". All species experience a germfree environment during prenatal development. Variations exist among different species at the time they enter the conventional environment, but all are endowed with some mechanism of defense against invading microbes. Amongst these is the acquisition of maternal antibody prior to birth or hatching, the detection and identification of which is facilitated in an animal subsequently maintained in the germfree or gnotobiotic state insofar as antibody produced by the gnotobiote's own immune mechanism is restricted to physiological stimulation, diet, inhalation and contact with dust, dander, etc. Antibodies were encountered in very young chicks in the present work. The antibodies are presumed to be IgG since antibodies of higher molecular weight are not transferred from the mother to the yolk. The germfree animal facilitates study of immune maturation in the absence of the most potent of exogenous stimuli, replicating microbes. This is particularly important when the test antigens share specificities with enteric microbes. A conventional animal's response to an antigen is not a primary response if the animal has been exposed to some of the antigen's specificities by contact with microbes. The best evidence of the importance of enteric microbes is the existence of natural antibodies.

In the past antibodies found in the sera of man and animals against bacterial agents without any deliberate immunization (see Topley and Wilson, 1955) could be plausibly explained by the carrier state, as established in the case of "natural" antitoxin production to diphtheria and scarlet fever, and by subclinical or latent infection by the microbe in question. In 1901, Paul Ehrlich termed substances capable of neutralizing toxins and of agglutinating and lysing bacteria and erythrocytes of the same and unrelated species as comprised of many "antiamboceptors" capable of attaching to receptors, "amboceptors", in such a fashion as to neutralize or block them. The heterogeneity of natural antibodies implied by this reasoning was confirmed much later by Owen (1954) who demonstrated that antibody populations (isohemagglutinins) from "normal" human subjects were fractionable by adsorption with cells of various animals.

Dupont had put forward the thesis in 1924 that all hemagglutinins arose through prior exposure to external substances of constitution similar to erythrocyte alloantigens. Finland and Curnen (1938) observed that an increase in antibody titre against human red cells of all four blood groups (A,B,O,AB) occurred in horses after immunization with type XIV pneumococci for the production of therapeutic sera. Use of these sera had resulted in unusually severe reactions in occasional patients receiving them, sometimes resulting in death. Subsequent adsorption with type XIV pneumococci removed all agglutinins to all four human blood groups.

Forssman's antigen, similar to human blood group A (Schiff and Adelberger, 1924) was shown to be contained in some infectious agents, the classical documentation being that by Paul and Bunnell (1932) of heterophile antibody in cases of infectious mononucleosis now known to be caused by a herpes-like (Epstein-Barr) virus, and by bacteria, including *Bacterium septicum* (Powell, H.M., 1926), *Bacillus dysenteriae* Shiga (Iijima, 1923, and Eisler as referred to by Wiener, 1943 and 1951) and *Diplococcus pneumoniae* (Bailey and Shorb, 1931, Eisler and Howard, 1932). Witebsky *et al*. (1935) found quantitative differences between pneumococcal types in their ability to inhibit hemolysis of sheep red cells by A-antisera. In the view of Wiener (1951) bacterial antibodies result from subclinical infections and natural hemagglutinins are most likely immune antibodies of heterogenetic microbial origin. This view was based, in part, on the work of Witebsky and his colleagues, as well as the findings of Goebel and associates (1938, 1939), who demonstrated the serologic and chemical relationships of blood group A substances, from commercial peptone and horse saliva, and that of "soluble specific substance" from type XIV pneumococci.

Springer (1956 and 1958) found a potent inhibition of blood group reactions in preparations of somatic polysaccharides in bacteria of various genera but particularly gram-negative species (none of the gram-positive organisms examined showed any blood group activity). For instance, *Escherichia coli* O_{86} exhibited high blood group B activity, *E. freundii* high blood group A activity, *Salmonella poona*, *Salmonella atlanta*, and *Salmonella grumpensis* group O activity. Adsorption of blood group agglutinins from different antisera with the above bacteria was accomplished in a specific fashion. General agreement was found between the nature of known carbohydrate building stones of blood group substances and the blood group activity of the investigated bacteria. That is, the terminal, non-reducing monosaccharides most responsible for serologic specificity were N-acetyl-D-galactosamine for A substance, D-galactose for B, and L-fucose for O.

This was extended to demonstrate that ingested plant and animal materials, but most particularly a viable microbial population in the alimentary tract of animals contributes largely to the acquisition by immunogenic means of anti-human blood group agglutinins (Springer *et al*., 1958, 1959, and 1961). White Leghorn chicks were divided into two groups; those kept in a conventional environment and those raised in a germfree tank and fed a semi-synthetic diet free of any demonstrable blood group activity (Springer *et al*., 1959). One-half the germfree chicks were fed once with live *E. coli* O_{86} at age 20 days. All chicks were tested for hemagglutinins to known B antigens on the 42nd and 66th days after hatching. Germfree chicks fed *E. coli* O_{86} exhibited a low but significant anti-B titre. Anti-human or anti-erythrocyte agglutinins which were completely removed by one adsorption with human O cells were formed in germfree chicks not fed *E. coli* at about the same age as in conventionally housed chicks but were consistently lower after about 30 days of life ~~and older~~. Specific blood group agglutinins were absent in these germfree chicks until 60 days whereas conventionally reared chicks could be demonstrated to possess them at 30 days of age.

In this report the study of natural immunizations is extended to chickens whose immune competence was intentionally altered and to chickens whose immune competence could be controlled by genetic selection.

Pollard, 1969, pointed out that the effects in mice of immunological impairment may be masked and distorted by the effects of their microbial flora. In the case of neonatal thymectomy (Wilson, Sjodin, and Bealmear, 1964, and Bealmear and Wilson, 1967) germfree thymectomized mice did not develop a wasting syndrome until conventionalized. In the graft-versus-host reaction (GVHR) as reported by van Bekkum, de Vries, and van der Waay, 1967, histologic lesions characteristic of the GVHR were present in mice given rat bone marrow cells following irradiation but the mice did not waste and die as did their conventional counterparts. Jones, Wilson and Bealmear, in 1971, demonstrated the GVHR resulting from allogeneic bone marrow transplantation was not necessarily fatal if the microbial flora of the host was restricted or eliminated.

It was with this type of phenomenon in mind that some of the work presented here was approached. It had been observed that in the genotypic strains $\underline{B}^{2}\underline{B}^{2}$, $\underline{B}^{2}\underline{B}^{14}$ and $\underline{B}^{14}\underline{B}^{14}$ of White Leghorns that hatchability, susceptibility to disease and survival (Sheridan *et al.*, 1969, and Longenecker *et al.*, 1972) were markedly lower in the $\underline{B}^{14}\underline{B}^{14}$ as opposed to the $\underline{B}^{2}\underline{B}^{14}$ and secondly to the $\underline{B}^{2}\underline{B}^{2}$ (the heterozygote being the most robust). At the same time the GVHR (Longenecker *et al.*, 1970 and 1972) was markedly increased in the F_2 $\underline{B}^{14}\underline{B}^{14}$ with a geometric progression from $\underline{B}^{2}\underline{B}^{2}$ through $B^{2}\underline{B}^{14}$ to $\underline{B}^{14}\underline{B}^{14}$ which was interpreted as being due to a gene dosage effect involving an increase in mitotic divisions of immunocompetent cells by one for each $\underline{B}^{14}$ allele. The question posed was whether or not the apparent genetic effect exhibited in increased histoincompatibility of the $\underline{B}^{14}\underline{B}^{14}$ was truly a genetic control by the histocompatibility locus in this case or whether it appeared to be so due to an inherited susceptability to microorganisms with a subsequent aberrant lymphoid stimulation resulting in a heightened reactivity in those tests measuring lymphoid function.

The same question of distortion of the immune response by the microbial environment could be posed in the case of extirpation of the two lymphoid organs, the thymus and the bursa of Fabricius. In particular, since chickens bursectomized on the day of hatching tend to show poor survival many immunological studies have not been done. Certain contradictions exist in regard to the nature of a delayed hypersensitivity reaction and its relationship to either bursal or thymus-dependent development.

This work was designed to test the effect of gnotobiosis on the cellular aspects of immunity, particularly those involving the GVHR in the chicken. Moreover, since cellular and humoral aspects of immunity are readily separable in this species by extirpation of the thymus and the bursa of Fabricius, re-examination of the effects of these unhindered by an indigenous microbial population seemed appropriate. One would not expect GVHR *per se* to be antigenically stimulated by microorganisms inhabiting the donor. Further, the system seemed conducive for the examination of the interaction or the lack thereof, of different types of cellular immunity such as delayed hypersensitivity and GVHR.

MATERIALS AND METHODS

A. Housing of Animals

1. Isolation Units

Isolation units or chambers of flexible plastic[1] 48 inches long by 24 inches wide by 24 inches high with fibreglass or stainless steel 12-inch diameter and 9-inch deep port on one side and eight mil frosted vinyl sleeves with gloves on the other were used. The inlet air filtering system consisted of asbestos filter material wrapped around a perforated stainless steel cylinder with an upper opening leading from the center of the cylinder into the isolator chamber. Below this opening which attached to the isolation chamber through a one-inch diameter nipple opening, the filtration unit was enclosed in a plastic sleeve with another opening for air driven in by a blower-motor of approximately 1/30 h.p. The outlet trap was a fibreglass cylinder with a sump to collect accidental overflow of trap contents and a float with an opening raised above the level of sterile mineral oil when the isolator was inflated and air was being driven through. If the isolator was deflated the float lowered so its opening was below the level of the mineral oil, thus preventing backflow of air. See Figure 1.

A second isolator, 42 inches long by 24 inches wide by 26 inches high, was used largely for hatching. It was built of wood, and subsequently fibreglassed to create a smooth, non-porous surface. A 40-inch by 18-inch slanted glass plate above the gloves in front allowed visualization of the interior of the chamber and another glass plate on the top of the unit helped provide additional lighting. A twelve-inch diameter, nine-inch deep stainless steel port with inside and outside plastic caps utilized for moving materials into and out of the isolator was built into the back of the chamber opposite the gloves. A four-inch diameter and nine-inch long cylinder fitted with a cap on its lower opening and two one-inch diameter holes near its top and bottom on opposite sides from each other used for spraying with peracetic acid to sterilize its interior was built into the lower right hand corner of the floor of the chamber to facilitate drawing eggs into the chamber. This was capped with a lid inside the isolation chamber. An inlet and outlet filter system was installed at one end with two filters at diagonal corners from each other. These consisted each of two stainless steel housing plates with two and one-half inch diameter opening to allow passage of air through the filter medium between the two housings which were clamped together by a grooved stainless steel screw clamp. A blower-motor similar to the above attached to the inlet housing forced air through the isolation unit. On the other end of the chamber, opposite from the filter systems, a hole fitted with a cork containing a bored hole served as an outlet for the electrical cord of the heating pad used to maintain the temperature inside the isolation unit adequate for hatching. See Figure 2.

Both types of isolation units were placed on tables whose top surfaces were constructed of a light stainless steel pressed wire mesh.

Inside the isolators the chickens were housed in eleven-inch diameter wire or aluminum mesh cylindrical cages, the bottom of which was raised one inch from the surface of the floor. All supplies, including cages, which could be autoclaved beforehand, were wrapped in brown paper and subjected to a minimum of one hour's autoclaving and taken into the isolator prior to its sterilization.

The inner surfaces of the isolation units and that of all equipment and supplies including wrapped and autoclaved items were sterilized by spraying with two percent peracetic acid, a strong oxidizing agent. A minimum of 48 hours was allowed to pass with air being circulated through each unit in order to drive out any residual peracetic acid or its fumes before sampling for sterility or before any animals or eggs were taken into the isolation chamber.

Testing for sterility was done by swabbing several areas, particularly the sites of any folds or corners, as well as some of the inside surfaces. The swabs were immediately inoculated into two tubes of Brewer's thioglycollate medium which were incubated at room temperature and at 37°C. in an attempt to screen out the majority of aerobic and anaerobic bacteria and fungi commonly encountered as aerial contaminants. The tubes were incubated a minimum of two weeks in the case of those at 37°C. and a minimum of three weeks in the case of those at room temperature before being called negative, although a presumptive negative decision was often made at 48 hours. Where possible the isolators were reswabbed prior to any animals or eggs being introduced.

2. Preparation of Eggs for Hatching

The preparation of sterile eggs for hatching was a modification of the original method described by Reyniers _et al_., 1949, (Luckey, 1963). Fresh, clean, fertile eggs were allowed to assume room temperature and were brushed vigorously in 0.1% hibitane (chlorhexidine diacetate powder)[3] solution at 38°C. Eggs were partially submerged in a shallow pan and about a dozen prepared at a time. Spent hibitane was discarded after each group of eggs and fresh warm solution was used for the next group. A clean brush was presoaked in hibitane before use. Eggs were then submerged under the surface in two percent mercuric chloride solution at 38°C. for five minutes. The mercuric chloride was allowed to dry on the shell on the premise that in the presence of sufficient moisture for spores to germinate or for bacterial replication, the germicide would also be activated. The eggs were placed in an incubator at 38°C. where they were rotated automatically every four hours until early on the twentieth day. At this time the eggs were candled. Active embryos were again brushed in warm hibitane solution as above to remove the mercuric chloride which is highly toxic to the chick on breaking through the shell. The embryos were placed in a nylon stocking presoaked in hibitane and drawn through a warm (38°C.) hibitane bath into the isolator. In the case of the solid isolator this was accomplished by reaching down the port or trap in the floor of the isolator which was submerged below the surface of the warm bacteriocidal agent in a tank and pulling the eggs into the isolator with the gloved hand. In the instance of the flexible isolator a plastic sleeve was attached to the port and the end away from the isolator submerged in warm hibitane. Its interior was sprayed with peracetic acid. Two people were required for the operation: one to submerge the eggs below the surface of the hibitane and bring them into the sleeve, and the other operator at the gloves opposite to grasp the stocking net with a hook and draw the eggs into the isolation unit. Originally, eggs were submerged for three minutes prior to drawing into the isolator but this was later found to be unnecessary from the point of view of maintaining sterility, and, since the procedure was, at the same time detrimental to the embryo, it was discontinued. During the procedures involving the handling of eggs outside the isolation chamber from the original cleaning to the point of entry into the isolator, sterile gloves or vinyl gloves whose outer surface had been treated with hibitane were worn. Inside the isolation chamber the eggs were spread along the bottom of shallow wire baskets which were then covered with aluminum foil. In the case of the flexible film isolators, infra red brooder lamps were placed under the isolator on the outside and electric heatpads were placed over the baskets inside in order to maintain the eggs at 38°C. for hatching. The heating pad only was used in the case of the solid isolator. Damp sponges were placed throughout the isolator to increase the humidity.

Once the embryonating eggs had dried off after being taken into the isolator, swabs of the outside surfaces were taken and inoculated into thioglycollate medium in an attempt to ensure that the procedure had been carried out sterilely. Also, on hatching, membranes remaining on the broken shells were cultured.

3. Preparation of Feed

Originally chick starter obtained from the Biosciences Animal Center, the University of Alberta, was used and simply autoclaved. Extra vitamins were added; fat soluble vitamins just prior to autoclaving and B vitamins in solution, sterilized by filtration, added after a ten milliliter quantity in a screwcap glass tube, sufficient for one kilogram of feed was taken into the isolator together with the autoclaved feed. The amount of the vitamins was that given for the preparation of a tryptophane-deficient diet prepared in the same manner. See below. Animals were kept on the chick starter for three weeks after which they were transferred to a tryptophane-deficient diet, the rationale being that since tryptophane is an essential amino acid for the chicken, then growth should be inhibited beginning with the time of transfer onto this diet. The purpose of doing so was for practical reasons only, namely, the space inside the isolators limited the number and size of animals which could be housed at any given time. It had been shown previously (Ruth, personal communication) that chicks on this diet from the time of hatching were still capable of mounting an immune response by producing antibody to different subgroups of the B blood group and histocompatibility antigens in the chicken. The tryptophane-deficient diet was made up in the following manner:

(a) Vitamin B solution prepared in 500 ml. volume:

+Riboflavin	250 mg.
Niacin	1500 mg. (1.5 gm.)
d-Ca. pantothenate	500 mg.
Choline (dihydrogen citrate)	75 gm.
++Folic acid	50 mg.
Thiamine	75 mg.
Pyridoxine	100 mg.
Vitamine B_{12}	500 ug.
+++Biotin	5 mg.

+Riboflavin was weighed out first and 50 to 100 ml. distilled water plus a few drops of glacial acetic acid added. If dissolution was unsuccessful, the solution was heated to the point of boiling.

++Folic acid was prepared separately in a concentration of 0.5 gm. per 100 ml. Sodium hydroxide was used to dissolve versus the acetic acid with riboflavin. Subsequently, ten ml. of this solution was used per 500 ml. of a B vitamin solution.

+++Biotin was also prepared separately in a similar manner to riboflavin. 0.1 gm. per 100 ml. proved a good concentration and five millilitres of this used per 500 ml. total volume of B vitamin solution.

Subsequently, all other of the above listed vitamins were added and the volume made up to 500 ml. This solution was then sterilized by millipore filtration and dispensed sterilely into sterile, screwcap culture tubes in ten millilitre amounts.

To this solution were added, aseptically, the following:

one millilitre vitamin A solution
0.5 ml. trace mineral suspension.

The latter two were prepared as follows:

(b) Preparation of Soluble Vitamin A solution from commercially purchased capsules:[4]

With a syringe and needle the capsules were punctured and the contents aspirated and dissolved in water such that one millilitre contained 2,000 U.S.P. units, the amount required per kilogram of feed. One millilitre of filter-sterilized solution was then added aseptically to the ten millilitres of vitamin B solution in a screwcap tube.

(c) Preparation of trace mineral mix (amounts given for 100 ml. of suspension):

manganous sulfate ($MnSO_4$)	25 gm.
zinc carbonate ($ZnCO_3$)	18 gm.

ferrous sulfate ($FeSO_4$)	8 gm.
potassium iodate	1 gm.
cobalt carbonate ($CoCO_3$)	0.2 gm.

The above were suspended in water and autoclaved one hour at 121°C. and 15 p.s.i. 0.5 ml. were added aseptically to the tubes containing the vitamin A and vitamin B solutions. In short, 0.5 ml. were added per kilogram of feed making a 0.5% concentration of these trace minerals per kilogram of feed.

(d) To assemble the tryptophane-deficient diet:

i. To one of two paper bags of 482 grams ground corn were added:

22.5	gm.	$CaHPO_4$	
16.0	gm.	$CaCO_3$	
2.5	gm.	NaCl	
4.0	mg.	menadione (vitamin K)	these were increased to 20 times this amount to compensate for denaturation during autoclaving (Luckey, 1963)
0.75	mg.	vitamin D_3	
0.02	gm.	vitamin E	

Two bags of 482 gm. corn plus these salts in one of them comprised one kilogram of diet.

ii. The bags were sealed with autoclave tape and autoclaved one hour at 121°C. and 15 p.s.i.

iii. After taking the feed into the isolator, one tube of the vitamins B and A and mineral mixture was added to two bags of corn (i.e., one bag of corn plus one of corn plus salts) and mixed thoroughly. It should be noted that it would have been equally acceptable to add the vitamins to a liter of autoclaved drinking water in the isolator.

For each batch of feed made up two spore strips, i.e., filter paper impregnated with spores of *Bacillus stearothermophilus*,[5] were inserted into two bags of feed. Following autoclaving the spore strips were removed and cultured in Brewer's thioglycollate medium for one week at 56°C. together with an unautoclaved control strip according to the instructions on the package.

Spore strips were included as controls in the autoclaving of all materials taken into the isolator, as well.

It was found that the chick starter was not always completely sterile after the autoclaving procedure and a synthetic diet similar to the tryptophane-deficient diet above but with 35% soybean protein added was substituted. This differed ~~only slightly~~ from diet C-8 of Forbes, 1959, in that it lacked ~~the~~ cornstarch, corn oil and copper sulfate. ~~of that diet~~. At a later date when this was also shown to be unsatisfactorily sterilized the method of sterilization was changed to gamma irradiation at a dosage of five megarads.

4. Monitoring for Sterility

Standard methods for detecting the presence of bacteria, fungi, and parasites were employed in monitoring the animals and isolators for the presence of organisms during the course of an experiment. No attempt was made, however, to monitor for the presence of viruses, rickettsia, mycoplasma (P.P.L.O.)

and there were limitations for detecting the presence of saprophytic Mycobacteria and some of the fastidious Bacteroidaceae. The following scheme was employed. Every week to ten days fecal samples were taken and inoculated to Brewer's thioglycollate medium and incubated at room temperature and 37°C. as above when checking the isolators. Additionally, every three to four weeks the samples were inoculated to blood agar plates incubated both aerobically and anaerobically (in a gas pak jar). Periodically, a phenethyl alcohol (P.E.A.) plate was also inoculated and incubated anaerobically in an attempt to facilitate the recovery of any _Bacteroides_ species according to the principle outlined by Dowell _et al_., 1964. Whenever possible, blood agar plates to be used for anaerobic incubation were placed in a gas pak jar for several hours prior to use to remove oxygen bound to the hemoglobin of the red cells in an attempt to achieve more prompt anaerobiosis after inoculation of the plates. All anaerobic plates were incubated a minimum of two weeks, and, wherever possible as long as three weeks. Aerobic blood plates were incubated a minimum of one week and thioglycollate media for three weeks and two weeks respectively for the room temperature and 37°C. tubes before being considered negative. From time to time a plate of Sabouraud's agar incubated at room temperature was also included in the regimen for detection of any fungi not cultivable on any of the above mentioned media or at those temperatures. At the time of moving animals and close to the termination of an experiment, fecal samples were subjected to the zinc sulfate flotation method to screen for the presence of cysts of intestinal protozoa and the remote possibility of helminth eggs. Animals which died were necropsied and the viscera, usually the liver, lungs and intestine were cultured in thioglycollate medium and on aerobic and anaerobic blood plates. Contaminating organisms recovered were identified according to genus and an attempt made to trace the source. Where practical they were identified further to the species level following standard bacteriological procedures by inoculating to differential media, and employing cultural and microscopic morphologic features.

5. Control Animals

Control animals in a conventional environment were given identical diets treated in the exact same manner as above with the exception of the first experiments involving the comparison of various parameters of the immune response of gnotobiotic and conventional $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ White Leghorn chickens, and those comparing the $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$, $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$, $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$ genotypes where an additional control group on the usual diet of 16.5% Lay Ration (Phase 2)[6] in a conventional environment was included. After initial experience with more pronounced cannibalism in conventional chickens on the tryptophane-deficient diet these animals were housed in separate small cages in the Biosciences Animal Center, Biological Sciences Building, the

University of Alberta. Those chickens on a conventional diet were housed in groups in specific rooms in the same animal center.

B. Effect of Gnotobiosis on Natural Immune Competence

Graft-versus-host reactivity, the development of antibodies to human blood group A, B and H(0) substances, weight gain, and length of survival after being subjected to the tryptophane-deficient diet were compared on 15 isolator homozygous $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ chickens put on the tryptophane-deficient diet at three weeks of age, ten conventional animals on the same diet, and five conventional animals on a conventional diet.

1. Graft-versus-Host Reactivity

Two procedures were carried out for measuring the graft-versus-host reaction (GVHR); firstly, that of enumerating focal opacities or pocks on the chorioallantoic membranes (Longenecker *et al.*, 1970, Coppleson and Michie, 1965 and 1966, Schierman and Nordskog, 1963, and Burnet and Boyer, 1961) of host embryos obtained commercially which involved a major *B* histoincompatibility with the isolator and conventional test animals, and secondly, measuring the embryo spleen weight using similar host embryos as with the pock assay.

For measuring pock formation, host embryos of random combinations but containing no known *B* alleles were incubated for twelve days at which time they were prepared for inoculation. The tip of the egg at the air sac was punctured with a needle. The side of the egg was painted at one spot with 2.5% iodine solution in 70% alcohol to disinfect the area as well as to serve as the site for a second puncture or slit through the shell and the shell membranes about midway along the long axis. With the egg held horizontally with this second puncture upward, suction was applied to the opening at the air sac and the chorioallantoic membrane (CAM) dropped to create an artificial air sac at the side of the egg. The eggs were laid along the long axis on cardboard egg trays, the opening on the side was sealed with melted paraffin and the eggs rotated 90°. The eggs were then recandled to observe for any visibly defective chorioallantoic membranes and the site of the new air space marked. An area of approximately one-half centimeter by one centimeter was scraped of its shell with a sawing wheel leaving the shell membrane. This was then covered with melted paraffin to expedite the removal of the shell membrane with forceps just prior to inoculation. The eggs were taped to the egg trays to maintain their position and the openings covered temporarily with masking tape. On removal of the tape 25 lambda of diluted whole blood were deposited on the CAM at two spots at opposite corners of the opening making a total inoculum of 50 lambda. At times a known weakly reactive sample was inoculated in the amount of 100 lambda. The tape was replaced and covered with melted paraffin to seal the opening. The trays with eggs were placed in the incubator taking care not to tilt them and incubated in this horizontal position for an additional four days.

On day 16 of incubation the embryos were killed and one-half the shell and the CAM including the area of inoculation were cut away and lifted off the embryo. Membranes from any dead or defective embryos were not included for counts. The inoculated membrane was removed from the shell with forceps and washed three times in cold water to rid it of excess blood and debris and placed in saline to soak. Ten or more eggs were treated in this manner for each blood sample to be tested. On counting the pocks, any membranes with large nodules of foci representing inadequate distribution of the inoculum were excluded as were those membranes with pocks located at the circumferential ring representing contact of the inoculum with the interface of the CAM and the inner shell membrane.

Both isolator and conventional donors were bled from the wing vein just prior to inoculation of the eggs using 10% sodium citrate as an anti-coagulant in an amount of ten percent of the total volume of blood to be collected. Dilutions of blood were made in Dulbecco's phosphate buffered saline (Dulbecco and Vogt, 1954) and were either 1/10, 2/10, or 3/10 of whole blood.

For measuring embryo spleen weight eggs were candled at 11 days incubation and a prominent blood vessel marked by pencilling a window of approximately four millimeters square over it. The shell was then sawed up to the shell membrane over this outline. The shell proper enclosed by the sawed edges was then lifted off with fine forceps and a drop of paraffin oil deposited on the shell membrane to render it transparent over the blood vessel. 0.1 ml. of whole blood was then injected into the vessel using a tuberculin syringe and 27 gauge needle. The window was covered and sealed with melted paraffin. Fifteen to 20 eggs were used per sample of blood wherever possible and incubated eight additional days. On the 19th day of incubation the embryos were killed, the sex of the host embryo determined by examining the gonads, and the spleens removed and weighed to the nearest milligram. Donor chickens were bled in the same manner as for enumerating CAM pocks.

2. Antibody to Human Blood Groups

Whole blood remaining after setting up either of the GVHR assays or that from separate bleedings was spun at 3,000 r.p.m. for 15 minutes to sediment the cells and the plasma removed. This was inactivated at 56°C. for 30 minutes and tested for the presence of anti-human blood group antibodies by transferring two drops of plasma with a tuberculin syringe fitted with a sawed-off 21 gauge needle and a rubber bulb to two wells in a microtitre plate. This "pipet" was rinsed three times in phosphate buffered saline (PBS) between samples. One drop of commercially prepared and standardized five percent human A_1, Rh-negative red blood cells was added to one well per sample and one drop of human B, Rh-negative to the other.[7] Additionally, titrations of control sera were set up as follows. Serial doubling dilutions in PBS of known human O plasma or serum, a known human AB, Rh-positive serum, a known negative sample of pooled chicken plasma, and, when available, a known positive sample of pooled chicken plasma were made from undiluted, one in two, and so on through 1/128. Two drops of each of the dilutions were transferred to two separate wells and a drop of A_1 and B cells added as with the whole chicken plasmas. A saline control of two drops of saline in each of two wells with a drop of A_1 and B cells was also included. The human O plasma served as a positive control of human antibody titre to blood group A and blood group B substances, the AB plasma or serum as a negative control as did the pooled chicken plasma. Sufficient known positively agglutinating chicken plasma was rarely available to pool and to run sufficient titrations on to determine the antibody titre. After the addition of the red blood cells the plates were tapped against the palm of the hand to suspend the cells evenly in the wells and placed on a Fisher rotator and rotated at 190 r.p.m. for 30 minutes at room temperature. The samples were then read for agglutination with the degree of agglutination designated from negative (-) to four plus (++++) with intermediate gradations. Four plus represented all cells agglutinated in one mass, one plus (+) represented a majority of free cells but visible clumps or granules of agglutinated cells present. A designation of plus or minus (±) was also included to indicate doubtful or very rare clumps or granules of agglutinated cells.

Any samples reacting positively were adsorbed with equal volumes of three times washed (in PBS) and packed human O, Rh-positive red blood cells to remove antibody to H(O) and species determinants for ten minutes, and twenty minutes successively in the manner outlined below for the determination of antibodies to human blood group substances. Occasionally, a third adsorption of forty minutes was necessary, and in the case of the conventional animals on a conventional diet, a fourth using a half volume of packed human O cells for one hour was required. The adsorbed plasmas were then titrated in the same manner as the positive and negative controls above. However, an additional row of serum dilutions was included and 5% washed human O cells added to them. Both adsorbed and unadsorbed sera were tested simultaneously.

3. Growth and Weight

Chickens were weighed on a plastic dietetic scale with a large pan in the isolators and another metal constructed scale was used for all conventional chickens. Weights were taken every week to ten days.

4. Survival

The time of death and the age of the individual animals was noted and the survival of conventional animals on the tryptophane-deficient diet compared with that of the gnotobiotic. Conventional chickens on conventional diet were eventually used for other experiments.

C. Determination of Antibodies to Human Blood Groups in Adult Conventional Chickens

Adult chickens of different histocompatibility and blood group genotypes were tested as were adult chickens which had had the superior cervical sympathetic ganglion (Alsager *et al*., 1971) on either right or left side or both removed on the day of hatching together with their sham and unoperated counterparts for the presence of antibodies against human blood groups.

Five millilitres of blood were taken from the wing vein using ten percent sodium citrate as an anticoagulant in an amount of 1/10 the total volume of blood collected. The blood was spun at 3,000 r.p.m. for 15 minutes and the plasma removed and inactivated for 30 minutes at 56°C.

1. Adsorption of Plasmas

One millilitre of inactivated plasma from each test sample to which an equal volume of three times washed in PBS human O, Rh-positive packed red blood cells had been added was incubated at room temperature for ten minutes with several mixings during this time. The samples were then spun at 3,000 r.p.m. for 15 minutes and the plasma removed. Another millilitre of packed cells was added to each plasma and the samples incubated for 20 minutes, spun, and the plasma removed. A third adsorption with one millilitre of packed cells was carried out for 40 minutes. The fourth adsorption involved an hour's incubation but using only 1/2 ml. of packed red cells. Occasionally, when this procedure did not remove all antibody to human H(O) blood group or species determinants, a fifth adsorption carried out in the same manner as the fourth was done.

2. Titration of Adsorbed and Unadsorbed Plasmas

Serial doubling dilutions of both adsorbed and unadsorbed portions of plasma from each animal were made by adding 0.2 ml. of undiluted plasma to 0.2 ml. of PBS, mixed, 0.2 ml. of this 1/2 dilution transferred to a second 0.2 ml. of saline, making a 1/4 dilution , and so on through to a 1/128 dilution. Two drops of each dilution were then transferred to each of three wells in a microtitre plate thus comprising three rows of wells for all dilutions. To one series of wells representing all of the dilutions of a given sample a drop of five percent A_1, Rh-negative cells (as above) was added to each well. To the second row a drop of B, Rh-negative cells was added and to the third a drop of five percent O, Rh-positive cells. In the case of the adsorbed sera the O cells provided an index of the effectiveness of the adsorption procedure and in the case of the unadsorbed portion of the plasma it was a test for the total reaction against all human red cell antigenic determinants. The microtitre plates were mixed as above, rotated for 1/2 hour at room temperature and read for agglutination in the same manner as described in comparing gnotobiotic and conventional $\underline{B}^2\underline{B}^2$ animals. Control positive and negative plasmas, as well as a saline control were included for the tests performed on any given day. The titre of each plasma, adsorbed and unadsorbed was taken as the highest dilution in which visible agglutination occurred. For statistical analysis an arbitrary designation of one was given in those instances where there was definite reaction in the undiluted serum only. A designation of two was given where the last dilution showing a reaction was the 1/2 dilution and so on to a designation of eight in the 1/128 dilution. Where the last reaction was a ±, 0.5 was added to the number which would have been designated to the previous dilution.

D. Effect of Gnotobiosis on Immune Competence of Chickens of Different Histocompatibility Antigens

Three different genotypes, $\underline{B}^{2}\underline{B}^{2}$, $\underline{B}^{2}\underline{B}^{14}$, $\underline{B}^{14}\underline{B}^{14}$, of White Leghorn chickens were hatched in isolator conditions and in the conventional environment. The animals were started off on the chick starter diet and transferred to the tryptophane-deficient diet at three and one-half weeks of age for the 30 isolator animals used and an equivalent number of conventional animals on the diet. Five birds of each genotype were maintained on the regular diet in conventional animal quarters.

1. Graft-versus-Host Reactivity

Graft-versus-host reactivity using the measurement of pock formation on the CAM (on hatching, where possible at the time of diet change, three months of age, and at five to six months of age) and the determination of increase in host embryo spleen weight (at six months of age only) were carried out as during the comparison of immune competency in $\underline{B}^{2}\underline{B}^{2}$ gnotobiotic and conventional chickens.

2. Antibody Against Human Blood Groups

Antibody to human blood group determinants were measured as before.

3. Weights of Animals

The animals were weighed at intervals throughout the experiment.

4. Antibody Production

A terminal experiment performed on these animals consisted of measuring the ability of the different groups to form antibody to sheep red cells (SRBC) and determining the number of antibody-forming cells using the Cunningham Plaque assay as modified by Fredericksen (personal communication). The animals were injected intravenously with 0.1 ml. containing 1 x 10^9 sheep red cells for every 100 grams of body weight. Five days later the animals were bled, sacrificed, and the spleens removed and prepared for the plaque assay. The plasmas collected were titrated with two percent SRBC.

E. Effect of Gnotobiosis on Immune Competency of Thymectomized Chickens

1. Isolation Procedure

The method of preparing embryos for taking into the isolation chambers was that described for the flexible isolation units. However, the embryonating eggs were taken into a flexible plastic isolation chamber modified for surgical procedures insofar as it had an 18 inch extension at one end which was ten inches deep and 24 inches wide (the width of the usual housing isolators used throughout these experiments). The top of this outpouching or extension was composed of a special see-through plastic appropriate for performing fine surgery. An additional set of gloves of lightweight vinyl were also attached to the extension. See Figure 3.

2. Surgical Procedure for Thymectomy

On the day of hatching the chicks were thymectomized. Anaesthesia was accomplished by injecting intraperitoneally 0.07 ml. of "Combutol" prepared by using three parts sodium pentothal in a concentration of 15 mg./cc. to one part of sodium pentabarbitol (Nembutal) in a concentration of 15 mg./cc. The anaesthetic was prepared immediately before use and taken into the isolator. It was not used beyond the day of preparation.

After the anaesthetic the feathers around the throat were wetted down with 95% alcohol and a long ventral incision made in the skin with double pointed scissors to a position caudal to the crop gland. The skin was pulled away from the incision on either side with fine, curved forceps. The fascia were dissected away on either side of the jugular vein near which the thymic lobes lie, the last lobe being deep behind the crop gland and under the clavicle just overlying the thyroid (Panigraphi, 1971). This, most caudal lobe, was lifted off first with fine, curved forceps and the more anterior lobes lifted off successively until the last, usually the seventh (although the numbers of lobes is not constant in the chicken - Panigraphi, 1971) was off. Thymic lobes were removed on both sides and the wound sutured or clipped taking care not to clip or suture the crop gland which necessarily had to be loosened during the surgical procedure. Isolator animals which died during the course of the experiment were examined macroscopically for thymic remnants. Any suspicious tissue was subjected to histological examination. Control animals in both environments consisted of sham operated animals subjected to the anaesthetic and the surgical procedure up to but not including the removal of the thymic lobes.

Animals were placed on the corn-soybean diet described earlier and maintained on it for four weeks before being transferred to the tryptophane-deficient diet.

3. Graft-versus-Host Reactivity

The enumeration of pocks on the CAM was carried out as described above. The first was performed at the age of four weeks.

Measurement of spleen weight was carried out just prior to conventionalization of the isolator animals using both embryos differing at the B histocompatibility locus (i.e., commercial random embryos) and incubated four days following injection, and identical at the B locus (i.e., both donors and recipients were $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$) and incubated eight additional days following injection. $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ fertile eggs were obtained from Hyline Poultry Farms.[8] These assays were repeated following conventionalization.

4. Conventionalization of Isolator Animals

After the first splenomegaly assay had been completed using host embryos of two different types as indicated above, the animals were removed from the isolation units and transferred to the regular animal quarters on the sixth floor animal center in the Biological Sciences Building at the University.

5. Antibody Against Human Blood Group

These antibodies were determined, as previously, on any remaining whole blood from bleedings for GVHR.

6. Growth and Weight

Animals were weighed periodically as previously.

F. Effect of Gnotobiosis on Immune Competency of Bursectomized Chickens

$\underline{B}^2\underline{B}^2$ eggs were prepared for taking into the isolator as previously and were taken into the surgical isolator unit. On the day of hatching the chicks were anaesthetized in the same manner as previously with the exception that the volume injected was 0.05 ml. inasmuch as the surgical procedure for bursectomy did not require a deep anaesthesia for as great a length of time.

1. Surgical Bursectomy

One-half the chicks hatched were surgically bursectomized by making a small incision above the cloaca. The tissues were then separated and stretched with forceps so as to expose the Bursa of Fabricius lying interiorly and connected to the inner cloacal membrane by its stalk. The membrane surrounding the bursa was stretched away with forceps. On complete freeing of the bursa it was possible to make it "burst" through the incision fully exposed. With a scalpel blade the ureters, at the side of the bursa were freed by gently scraping along the sides of the bursa between it and the ureters and adjacent blood vessels. The bursa was freed at its stalk by gentle scraping with the scalpel up to the point where it could be cut free with one small stroke. The wound was neither clipped nor sutured. One-half of the birds were sham operated by subjecting them to the anaesthesia and carrying through the surgical procedure up to the point of but not including removal of the bursa.

2. Chemical Bursectomy

Newly hatched chicks were treated with cyclophosphamide (in Canada, Procytox)[9] in a regimen of injecting two milligrams on four successive days beginning with the day of hatching (Linna, Frommel, and Good, 1972, and Toivanen, Toivanen and Good, 1972). The above germfree animals and their conventional counterparts were sub-divided into two more groups, i.e., four altogether: sham operated treated with cyclophosphamide by intraperitoneal injection according to the above schedule, and sham operated only; surgically bursectomized treated with cyclophosphamide, and surgically bursectomized only.

The animals were placed on gamma-irradiated at a dosage of five megarads corn-soybean diet and maintained on this for eight weeks and then transferred to the tryptophane-deficient diet.

3. Graft-versus-Host Reactivity

This was measured using the enumeration of pocks on the CAM at five weeks of age, at eight weeks of age, at the time of transfer to the tryptophane-deficient diet and three weeks after having been given BCG vaccine, at nine weeks of age following skin-testing of birds with mammalian purified protein derivative (P.P.D.) and again at sixteen and seventeen weeks of age at the time of skin-testing with crude BCG.

4. Sensitization with Mycobacteria

At age about five and one-half weeks animals out of each of the groups were given BCG (Baccilus of Calmette and Gúerin) vaccine[10] in an amount of two milligrams injected into the pectoral muscle. A period of two and one-half to three weeks was allowed for the development of the hoped for hypersensitive state, at which time the animals were skin-tested. Isolator animals given BCG were housed in separate isolators from those which had not been given BCG.

5. Testing for Hypersensitivity

0.1 ml. of mammalian purified protein derivative (mantoux) of five T.U. (tuberculin units) per 0.1 ml.[11] was injected intracutaneously into the wing web between the humerus and radius of one wing. The other wing served as a control. The thickness of both wing webs was measured prior to injection, at 2, 4, 8, 12, 24, and 48 hours post injection with a micrometer. The measurement prior to the injection of P.P.D. served as an additional control.

Seven weeks later skin testing was repeated by injecting 0.1 mg. crude BCG into one wing web as above. Skin thickness measurements were taken at 5, 24, 48 and 72 hours.

6. Capillary and Ouchterlony Precipitation Tests

Any remaining blood from each bleeding in the above indicated schedule for GVHR was spun to sediment the cells and the plasma removed and stored for future testing for possible humoral antibody against tuberculin products. For capillary precipitation each specimen was set up in serial 1/10 dilutions from 0 to 1/1,000 in phosphate buffered saline (PBS) using drops delivered with a pasteur pipet. Mammalian P.P.D. was drawn up into a capillary tube to one-third its length followed by an equal volume of plasma dilutions. The tubes were then inserted into modelling clay and incubated at room temperature for one hour, observing for precipitation at intervals throughout that time.

Ouchterlony plates were prepared by dissolving 1% Nobel agar in 0.15 M. NaCl and 1.5 M NaCl (Anderson, 1972). Wells were punched in the agar in a pattern of one central well with five surrounding wells equidistant from the central one. Each undiluted plasma was tested against mammalian P.P.D. in the central well. Plates were observed for lines of precipitation over a period of seven days.

7. Cunningham Plaque Assay for Antibody Producing Cells

To determine the effectiveness of both the surgical and chemical bursectomy the ability of the animals to mount a primary immune response to sheep red blood cells (SRBC) was measured by the Cunningham method of enumeration of antibody-producing cells from the spleen and worked out for the chicken by Fredericksen (personal communication). The animals were bled from the wing vein for basic determination of "natural" antibodies to SRBC following which all were injected with a standard concentration of 5 x 10^8 SRBC washed three times in PBS without calcium and magnesium ions. The animals were weighed at this time in the case of the conventional birds and just prior to a final bleeding in the case of the isolator birds. A final bleeding was performed five days later by cardiac puncture and the animals terminated immediately thereafter. The spleens were removed using "clean" technique, placed in PBS, and subsequently weighed. A small portion of each spleen was removed for histological processing and observation. The capsule was removed from the remainder of the spleen which was then pressed through a syringe fitted with a wire mesh and 21 gauge needle into PBS (again without calcium and magnesium ions) with ten percent fetal calf serum. Tubes were kept cold by embedding in ice. The cells were spun down and resuspended such that the estimated dilution approximated 1,000 lymphocytes per 0.1 ml. Cell counts were performed on each sample and where necessary the suspension was diluted to contain 10^5 spleen cells per milliliter. 0.1 ml. of this suspension was added to 0.075 ml. 10% fetal calf serum (FCS). A small drop (approximately 0.025 ml.) of 20% washed SRBC was added as well as 0.025 ml. containing two units of previously titrated chicken complement. The mixture was placed between Cunningham slides, the edges of which were sealed by dipping into a molten mixture of a 1:1 proportion of paraffin and Amojell, and incubated for one hour at 37°C. Small zones of lysed SRBC (plaques) were then counted under the dissecting microscope.

8. Titration of Plasmas for Determination of Antibody Against Sheep Red Blood Cells

Serial doubling dilutions of heat inactivated (56°C. for 30 minutes) plasmas collected prior to the injection of SRBC and five days following were made. 0.025 ml. of two percent SRBC were added to 0.025 ml. of each plasma dilution in a microtitre plate, mixed and incubated two hours at 37°C. Agglutination was read from negative to four-plus as with the ABH(O) titrations above.

9. Acquisition of Antibody Against Human Blood Groups

Measurements were carried out as previously described for other groups of experimental chickens.

FOOTNOTES FOR MATERIAL AND METHODS

[1] Obtainable from Standard Safety Supply Co. Ltd., Germfree Supply Division, Palatine, Illinois.

[2] Obtainable from F.M.C. Chemical Co., Buffalo, New York.

[3] Available from Ayerst Laboratories, Division of Ayerst, McKenna and Harrison, Ltd., Calgary, Alberta.

[4] Aquasol A, Arlington Laboratories, Montreal, Canada, and usually available in local drug stores.

[5] Prepared by Scientific Products and available through Canadian Laboratory Supplies Ltd., Canada.

[6] 16.5% Lay Ration (Phase 2). Available from Northwest or Co-op Feeds.

[7] Obtained suspended in modified Alsever's solution with inosine and disodium E.D.T.A. and preserved with Neomycin sulfate, 1:10,000 and Chloramphenicol, 1:3,000 from Ortho Diagnostic Laboratories, Don Mills, Ontario.

[8] Hyline Poultry Farms, Johnston, Iowa.

[9] Available from Frank W. Horner Ltd., Montreal, Quebec.

[10] Obtainable from Connaught Medical Research Laboratories, Toronto, Ontario.

[11] Obtainable from Connaught Medical Research Laboratories, Toronto, Ontario.

RESULTS

A. Housing of Animals

1. Preparation of Eggs for Hatching

Three treatments were tested: (1) spraying of the eggs with 2 percent peracetic acid on the 20th day of incubation just before transfer to the germfree isolator, (2) submersion of the eggs in hibitane at 38°C for 10 minutes before setting the eggs, followed by submersion in hibitane just before transfer to the germfree isolator, and (3) submersion in mercuric chloride at 38°C for 10 minutes before setting the eggs, followed by submersion in hibitane at 38°C for 10 minutes just before transfer to the germfree isolator. Treatment 1 gave the greatest hatchability, and since no contamination was observed treatment 1 was retested by bringing a second group of embryonated eggs into the isolator. This time it proved to be an inadequate means of sterilization as evidenced by the recovery of _Staphylococcus epidermidis_ and a _Micrococcus_ sp. In consequence, another method of preparing eggs was adopted as outlined in Materials and Methods. This is a modification of the other two procedures and sacrifices a certain amount of viability of the embryonated eggs for a safe method of sterilization. The hatchlings derived from these initial procedures were those used for the initial comparison of the effect of gnotobiosis on the natural immune response of $\underline{B}^2\underline{B}^2$ chickens. Tables 1, 2 and 3 give the hatchability for the three subsequent experiments determining the effects of gnotobiosis on different genotypic strains, thymectomized $\underline{B}^2\underline{B}^2$ chickens and bursectomized $\underline{B}^2\underline{B}^2$ chickens respectively. In these three groups no contamination could be detected inasmuch as no organisms were isolated from swabs taken from eggs immediately following entry into the isolator, from shell membranes, or from fecal samples within 24 to 48 hours after hatching when inoculated into the screening media outlined in Materials and Methods. This permitted the definition of any future contamination within the isolation facilities as being from other sources. The Clostridium perfringens and the gram-negative aerobe also isolated from the first group of $\underline{B}^2\underline{B}^2$ chickens (Table 1M, Appendix) but recovered later were traceable to inadequately sterilized feed from which they were also recovered. These organisms also existed in the initial untreated feed.

2. Monitoring for Sterility

Any organisms recovered on the screening medium either initially or throughout the course of the maintenance of a given group of experimental animals were subjected to differential media to further identify the organisms. Tables 1M through 4M in the Appendix give the organisms recovered from isolators throughout the course of the maintenance of the initial $\underline{B}^2\underline{B}^2$ groups of animals, $\underline{B}^2\underline{B}^2$, $\underline{B}^2\underline{B}^{14}$ and $\underline{B}^{14}\underline{B}^{14}$

genotypes, thymectomized and sham operated $\underline{B}^2\underline{B}^2$'s and bursectomized and sham-operated $\underline{B}^2\underline{B}^2$'s respectively. The results from differential media are given as supportive evidence for the identity of a given organism. They include colonial and microscopic morphology, results of fermentative or oxidative utilization of carbohydrates, any tests for the presence or absence of characteristic enzymes, oxygen requirements and other cultural characteristics.

It should be noted that with the exception of the final bursectomy experiment autoclaving was used as a means of sterilization of the feed. In these instances Clostridia, Bacillus species, fungi (*Scopulariopsis* sp. in particular) were recurrent contaminants, all of them being sporulating organisms and highly resistant to heat treatment. All were consistently recoverable from both the autoclaved and untreated feed. The Clostridia are gram-positive sporulating anaerobes, recoverable only in media with reduced oxygen tension; *Bacillus* sp. are gram-positive sporulating aerobes. The problem of this type of contamination was ultimately resolved through sterilization of the feed by irradiation thereby reducing contamination hazards to human error. The recovery of *Staphylococcus epidermidis*, *Micrococcus* sp., *Corynebacterium* sp. were consistently related to errors in the observation of sterile technique, or, as in the case of the use of 2% peracetic acid in sterilization of embryonated eggs, failure of the sterilizing agent under the circumstances in which it was used.

B. The Effect of Gnotobiosis on Natural Immune Competence

1. Graft-versus-Host Reactivity

The effect of gnotobiosis on GVH competence was examined by comparing chickens raised in a gnotobiotic environment with others raised in a conventional environment. The isolators which housed the gnotobiotic chickens were monitored for bacterial contamination throughout the course of the experiment (Materials and Methods) with the sequence and nature of the organisms recovered given in Table 1M. The gnotobiotic chickens were fed a tryptophane-deficient diet after the first three weeks of life in order to restrain their growth and prevent overcrowding in the isolators. A second group of chickens, maintained in a conventional environment, received the same diet. A third group, maintained in a conventional environment, received a normal tryptophane-rich diet. There were 12 gnotobiotic chickens and 7 conventional chickens on the tryptophane-deficient diet, and 5 conventional chickens on the normal diet. One gnotobiotic chicken died at the age of 6 days without evidence of infection, and the remaining 11 survived the experiment. One conventional chicken on the deficient diet died at the age of 42 days, and this was followed by other deaths such that only 2 of these chickens survived the entire experiment. These chickens were housed together and suffered greatly from cannibalism, presumably induced by the deficiency in their diet. All of the conventional chickens on the normal diet survived the experiment (Figure 7). Tryptophane-deficient chickens reached about 90 grams at the end of the first month with a slight increase in weight thereafter to around 100 grams. Normal-diet chickens continued to grow throughout the course of the experiment (Figure 6).

The GVH competence of these chickens was measured in two ways: by counting the number of graft-versus-host (GVH) pocks which appeared on the chorioallantoic membrane (CAM) of outbred embryos four days after inoculation of the CAM with peripheral blood, and by weighing the spleens of outbred embryos 8 days after intravenous injection of peripheral blood. The embryo data were grouped according to the age and treatment of the donors used in the CAM pock tests and according to the age, treatment, and sex of the recipient embryos used in the splenomegaly tests, and subjected to analysis of variance. The CAM pock counts of embryos inoculated with cells from gnotobiotic donors were compared with the pock counts of embryos inoculated with cells from conventionally housed donors maintained on the same, deficient diet. The conventional environment increased GVH competence, expressed as CAM pocks ($p < 0.025$). The increase in the number of CAM pocks, with the age of the donors between six weeks and 4.5 months (Figure 4 and Table 4), was highly significant ($p < 0.005$) albeit these chickens did not grow during this period (Figure 6). The conventionally housed chickens maintained on a normal diet were markedly more competent than the conventionally housed chickens whose growth was restrained by the deficient diet ($p << 0.001$). See Figure 4. The differences were less when tested by GVH splenomegaly (MES = mg. embryo spleen), and they varied depending on the sex of the recipient embryo. The female embryos which received intravenous injections of cells had larger spleens than male embryos which received the same injections (in contrast spleen weights of male controls were larger than female controls, Table 4). The effects of diet and environment on GVH splenomegaly were modest ($p < 0.025$) and the age of the donor had little if any effect ($p < 0.100$). The effect of recipient embryo sex on splenomegaly was very highly significant ($p < 0.001$). The CAM pocks were obtained from 630 embryos and the spleen weights were taken from 362 embryos. All available donors were used for each test of age and treatment, the minimum number of donors representing a given group being the two surviving conventional chickens on the deficient diet for the final CAM pock test. The mean for all embryos used in a test did not differ materially from the mean obtained by grouping embryos according to the individual donor, taking the mean of each of these groups, and averaging these "individual donor" means. It is clear that the more sensitive of the GVH assays, the CAM pock test, whose replicability was tested, Table 4, is affected by the environment to which the donor is exposed. The gnotobiotic chicken's GVH competence, measured by this assay, increases with age, but does not attain the level of competence displayed by the conventionally housed chicken during the first four months of life.

The superior GVH competence of conventionally housed chickens, as compared with that of gnotobiotic chickens, may represent a general stimulation of immune activities by bacteria. Or, it may represent a specific stimulation of those immune activities which are of particular importance in CAM pock tests. The latter interpretation implies that some of the invading bacteria share the antigenic specificities of the histocompatibility antigens of the chicken. If this is the correct interpretation we might expect to find a parallel between the acquisition of GVH competence and the natural immune response to bacterial antigens. The latter can be assessed, in part, by testing the blood for the presence of antibodies which agglutinate human erythrocytes, since these share antigen specificities with some of the conventional chicken's microflora.

2. Antibody to Human Blood Groups

Three kinds of antibodies can be readily discriminated through the use of human erythrocytes: antibodies to human A_1 and B antigens and antibodies to other erythrocyte antigens characteristic of the species but the nature of which is not known. These are referred to here as O antigens because they can be demonstrated with human O erythrocytes. The chickens used in the GVH tests were tested for the presence of antibodies to the human A_1, B, and O antigens. Gnotobiotic chickens did not have detectable levels of antibody. Conventionally housed chickens maintained on the deficient diet produced antibodies to human B and O antigens. Conventionally housed chickens maintained on the normal diet produced antibodies to human A_1, B, and O antigens. The levels reached a peak at about three to four months of age and then declined (Figure 5). By seven months the levels of antibodies to B and O antigens had recovered and antibodies to A antigen had reappeared. This is in marked contrast to the progressive increase in GVH competence which occurred in the same chickens over the same experimental period. Although the level of GVH competence is increased by exposure of the chicken to a conventional environment, the exposure does not explain the progressive rise in GVH competence, which parallels that of gnotobiotic chickens, and it does not vary in parallel with the chicken's natural antibody response to some of the resident bacteria.

All animals were tested periodically prior to the detection of appreciable amounts of antibody. In three individuals there was the suggestion that maternal antibody had been passed on to the young inasmuch as plasmas from these reacted weakly with human O cells at ages one and nine days post hatching. Insufficient plasma to adsorb with human O cells prevented determination of antibody specific for human blood groups A_1 and B.

3. Growth and Weight

In addition to the growth characteristics noted above (between the three groups of experimental animals) it should be pointed out that those animals whose growth was restrained by the tryptophane-deficient diet, on which they were placed at three weeks of age, exhibited a decreased weight as early as seven to nine days of age (Figure 6). This implies a deficiency in the sterile feed beyond the deficiency of tryptophane not compensated by vitamin supplementation.

4. Survival

The life span in days of individual chickens and the duration of the experiment is given in Table 5, in support of Figure 7.

C. Determination of Antibodies to Human Blood Groups in Adult White Leghorn Chickens

All spot tests as well as all titrations for antibody against A_1 and B, Rh-negative cells were accompanied by controls in which the agglutinability of the A_1 and B cells was demonstrated by agglutination with plasma from human O, Rh-positive donors since such plasma should contain antibody to both blood groups, A and B. The plasma was frozen in 0.5 ml. aliquots and one of these was thawed just prior to use. In order to characterize somewhat the nature of the variation and the reproducibility of the test the range, coefficient of variation and the standard error were determined for the reaction with A_1 and B, Rh-negative cells for each of two plasmas. Table 6 represents titrations done on two different plasmas collected from outdated human O, Rh-positive blood obtained from the University of Alberta Hospital Blood Bank Laboratory and which had been previously typed by the Canadian Red Cross Blood Transfusion Service.

Titres to A_1, Rh-negative and B, Rh-negative cells were more or less similar in the first plasma, as were titres to B, Rh-negative cells in both of the two plasmas. The titre of antibody to A_1, Rh-negative cells was high in plasma #2, differing from its own antibody to B cells and from the first plasma's antibody titre to A_1 cells. The range of end points was particularly high in the second plasma's reaction to B, Rh-negative cells (1.5 to 7.5), having a coefficient of variation of 21.8% as compared with 5.3% of the same plasma's coefficient of variation to A_1, Rh-negative cells. The coefficients of variation to A_1, Rh-negative and B, Rh-negative cells were 16% and 11.9% respectively for the first plasma, implying a relatively insensitive test. However, standard errors were somewhat more encouraging. No effect on titres with storage could be observed.

Moreover, to characterize the system more closely in the initial phases, plasmas taken from adult, conventionally housed, chickens selected randomly and without knowledge of genotype, age or previous history were titrated following storage for different times, nine days, two months, four months and five months as well as upon rebleeding. In no instance was there a significant difference in titre to either human A_1, Rh-negative, B, Rh-negative or O, Rh-positive human red cells following storage at any of the times indicated. Adsorption (four times with human O, Rh-positive cells) did not influence the stability of titres of antibody to A_1, and B, Rh-negative cells for nine days which were the only duplicate titrations performed on the limited quantities of adsorbed plasmas available. Moreover, although individual differences existed in the titre of anti-A_1 versus the titre of anti-B, the four individuals used in this experiment did not indicate a predilection for the formation of antibody to one particular blood group over the other. Those animals from which it was possible to collect sufficient plasma for storage over a period of time were also available for rebleeding and for testing the cells purchased from Ortho Diagnostics just prior to the three-week expiry date with those just received. No difference could be determined in the reactivity of the older versus the newer cells with the same plasma(s). No appreciable difference could be detected in plasma from a fresh bleeding when compared with stored plasma from the same animal. Unadsorbed plasmas only were used for comparison.

Adsorption of plasmas with O, Rh-positive cells such that no reaction with O cells could be demonstrated decreased the titre of antibody against A_1, Rh-negative human cells as well as to B, Rh-negative cells.

To determine whether or not a great difference existed in the titre of antibodies to human red cells generally in adult, conventionally housed White Leghorns, or more specifically to A_1, Rh-negative or B, Rh-negative cells different genotypic chickens were bled. The plasmas were adsorbed three or four times with O, Rh-positive cells to remove antibodies to O and to other unknown species determinants and were subsequently titrated for antibody remaining to A_1 and B blood groups. Genotypes included $\underline{B^{2}B^{2}}$, $\underline{B^{2}B^{14}}$, $\underline{B^{14}B^{14}}$, $\underline{B^{13}B^{13}}$, $\underline{B^{2}B^{15}}$ and homozygous and heterozygous $\underline{B^{21}}$. Table 7 gives the arithmetic designation for end-points obtained. Mean, range, and standard error are indicated for each group of titrations against a given cell type or blood group. There was an overall tendency toward greater antibody titres to B as exhibited by the mean. Differences were apparent between individuals. Antibody to O could not be demonstrated to be different in quantity from that reacting with A_1 and B, Rh-negative cells in the unadsorbed plasma. As before, a marked decrease in antibody to A_1 and B, Rh-negative cells remaining after adsorption with O, Rh-positive cells could be demonstrated.

Ganglionectomy at hatching (Alsager et al., 1971) affected the ability of male chickens to mount an immune response in the form of antibody production, following "normal" physiological stimulation, to human O, Rh-positive and other erythrocyte antigens characteristic of the species but not definable in the ABH(O) system. Figure 8 illustrates the difference in antibody titre of unadsorbed plasmas in male and female ganglionectomized and sham operated chickens to human blood cells of groups A_1, and B, Rh-negative and O, Rh-positive. There was no significant difference in the level of reactivity to the three different cell types among females alone or males alone as would be expected since all cells would detect both their specific blood group antibody as well as antibody to those unknown antigens characteristic of the species. In each treatment group the titres from plasmas of male chickens were less than those of the females. This was most pronounced where the right ganglion, or both right and left ganglia had been removed. Males with the right and left ganglia removed differed from all other groups insofar as a definite progressive increase in titre in the unadsorbed plasma could be demonstrated from that to A_1 cells through B cells to the highest titre against

O cells. There was a tendency toward the same progression of increase in titres amongst the females in this and the sham operated group.

Amongst the males, the results suggest that removal of the right ganglion had a more pronounced effect since antibody to cells of the three blood groups was markedly less than in the groups which were sham operated or in which only the left ganglion was removed. Removal of both right and left ganglia effected a decrease in antibody titre even further with the antibody titres in this group being less than that in all three other groups. These observations can be interpreted only with respect to some antigenic component(s), the nature of which is unknown but can be demonstrated with three different human red cell types. Thus, it would seem reasonable to think that the female reacts more strongly to some heterogenetic component(s) or antigen(s) than does the male. This difference is intensified by removal of the right superior sympathetic ganglion. The difference could not be demonstrated to be specific within the ABH(O) blood group system.

Adsorption of these plasmas with O, Rh-positive human red cells obliterated the difference between the sexes in antibody reacting with A_1, and B, Rh-negative cells. In no case could an appreciable difference between the sexes be shown. In the sham operated there appeared to be a reverse tendency with the males acquiring a greater antibody titre than the females to A_1, Rh-negative cells. See Figure 9.

Although the overall level of specific antibody to A_1, and B, Rh-negative cells seemed less in the group with both right and left ganglia removed than in the other three groups, this was not great; nor were the differences between the other three groups great. Specific antibody titre to A_1, Rh-negative and B, Rh-negative cells was reduced in the adsorbed plasmas to about one-half the titre of reactivity in the unadsorbed plasmas, similar to results with other adult chickens above.

D. Effect of Gnotobiosis on Immune Competence of White Leghorn Chickens of Different Genotypic Histocompatibility Antigens

1. Graft-versus-Host Reactivity

Since it had been noted in the previous experiment involving the examination of the effect of gnotobiosis on GVH competence of chickens of $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ genotype that diet had an effect more pronounced than microbial status an initial attempt was made to determine the effects of the change to a tryptophane-deficient diet on the GVH response. Gnotobiotic and conventionally housed donors of three different genotypes, $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$, $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$, and $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$, were used in CAMP assay at ages three to seven days, three to four weeks of age, namely, at the time of diet change, and at three months. The analysis of variance was done with adjusted n values (i.e., according to the lowest value of n for a given group) since a great disparity involving the number of donors, hence the number of recipient embryos used per genotype at any given age existed. Figure 10 shows the correlation of conventional CAMP with that of gnotobiotic (Gn) CAMP when the mean $\log_{10}$ values of the former are plotted against the mean $\log_{10}$ values of the latter. Table 8 gives the mean $\log_{10}$ values at the different ages of the three genotypes including that of the total sum. Values were higher in the conventional environment. However, on application of the F-test, this was found to be non-significant, namely, p = 3.1602 which is non-significant at the 5% level where $F_{1,270}$ represents the degrees of freedom of the variance ratio. The slope of the correlation curve is 0.94 where conventional values are plotted on the ordinate and gnotobiotic values on the abscissa, indicating a parallel acquisition of GVH competence between gnotobiotic and conventional chickens of all three genotypes. Figure 11 gives the mean CAMP of the three genotypic donors at different ages. It should be noted that at age three to four weeks when the animals were transferred to the tryptophane-deficient diet GVHR was at its highest level. The diet may be the influencing factor in the drop in GVHR competence at three months which then increased again in the $\underline{B}^{2}\underline{B}^{2}$ and $\underline{B}^{14}\underline{B}^{14}$. The order of GVHR competence of the three genotypes was $\underline{B}^{14}\underline{B}^{14} > \underline{B}^{2}\underline{B}^{14} > \underline{B}^{2}\underline{B}^{2}$. The increase in GVH competence between three and six months of age supports the hypothesis that the immune mechanism matures (at least to some extent) in spite of dietary restrictions. This is not necessarily in phase with the growth of the animal. Table 17 indicates that the animals grew slightly between the time of diet change and the termination of animals and did not experience the same decline in weight at three months as in GVH competence.

Difficulties had been experienced throughout the course of this investigation with the source of eggs used as hosts. Hence, a repeat experiment was performed on all donor animals living at six months of age at which time conventional animals on a conventional diet were included in the experimental protocol. Both of the previous assays for GVHR, namely, the CAMP assay and the splenomegaly (MES) were employed. Replicate analyses for both assays were performed on some of the donors on tryptophane-deficient diet where the experiments had to be performed over a period of more than one week involving embryos incubated at different times. Paired analysis of variance was carried out. Table 9 shows the mean $\log_{10}$ CAMP and MES values of replicate tests of gnotobiotic and conventional animals of the three genotypes. Figure 12 illustrates the combined gnotobiotic and conventional replicates. At this age the environment had a significant effect, an observation noted previously with $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ donors. A total of 221 recipient embryos was involved in the paired CAMP tests. The conventional donors were more competent ($0.001 < p < 0.025$). See Table 9. The genotypic effect was more marked ($p < 0.001$). The order of GVH competence was again $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}} > \underline{B}^{\underline{2}}\underline{B}^{\underline{14}} > \underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ except in the case of the gnotobiotic where $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$ were slightly lower than $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$. In the splenomegaly analysis it was not possible to include all genotypes of conventional donors for replicate tests. A total of 79 embryos was involved in this case with five individual donors. In every case the second test gave a lower MES than did the first (Table 9). Application of the F-test indicated significance implying that different groups of recipient embryos could give varying results at different times. Although only gnotobiotic genotypes could be compared, $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$ were again higher than either $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ or $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$.

On the overall analysis of variance embryo data were grouped according to genotype and treatment of the donors used for the CAMP test, and according to genotype, treatment, and sex of the recipient embryos used in the splenomegaly test. The effect of normal diet and conventional environment, expressed as CAMP pocks, was to increase GVH competence significantly ($p < 0.001$). A total of 588 recipient embryos was involved. Conventional donors on conventional diet were five each of $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ and $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$ and three of $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$. There were two conventional $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ donors on the tryptophane-deficient diet, four $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$ and three $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$. Gnotobiotic donors on the tryptophane-deficient diet were: five $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$, six $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$ and seven $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$. The order of donor competence was conventional on conventional diet > conventional on tryptophane-deficient diet > gnotobiotic on tryptophane-deficient diet. $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$ were more competent than $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ which in turn appeared to be slightly more competent than $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$. However, in no case could this latter difference be shown to be significant on application of the F-test. Table 10 gives the mean $\log_{10}$ values of CAMP for the three genotypes in different environments and on different diets. Significant interaction, $F_{4,579} = 5.0896$, $p < 0.001$ was observed. In an attempt to define the source(s) of interaction data were regrouped according to the type of diet rather than the environment (since it was apparent that the diet had a profound effect on donor efficiency) and according to the presence or absence of the $\underline{B}^{\underline{2}}$ allele. The tendency toward lesser GVHR competence in the $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$ as compared with $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ for all treatments was surprising in view of the work of Longenecker _et al_. (1972,1973). Table 10b gives the mean $\log_{10}$ of the values of CAMP of donor animals on the tryptophane-deficient diet (both gnotobiotic and conventional) versus those on conventional diet, as well as the overall total mean. The dietary effect was very marked: those animals on the deficient diet being much less competent in GVHR ($p < 0.001$). $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$ donors were still the least competent of the three genotypes in each case. Moreover, the same pattern held when data were grouped according to the gnotobiotic environment as opposed to the conventional, Table 10c. Possible reasons for this observation are discussed later.

On grouping the data for $\log_{10}$ CAMP where the presence of the $\underline{B}^{\underline{2}}$ allele existed versus its absence, Table 10d, the donors with the $\underline{B}^{\underline{2}}$ were markedly less competent than those without. Conversely, where data were grouped for the presence of the $\underline{B}^{\underline{14}}$ allele in the donors versus its absence, the greater competence of these donors was still highly significant ($p < 0.001$). See Table 10e. In both cases, the environmental and dietary (indicated as treatment) effect remained of the same order as previously and was again highly significant. As in the previous experiment, the mean for all embryos used did not differ materially from the mean obtained by grouping embryos according to the individual donor (compare Figure 14 with Figure 13). All CAMP values are for 0.1 ml. whole blood.

GVH competence at six months of age of the three genotypes, both CAMP and MES (according to recipient embryo sex) are shown in Figure 13.

The effects of environment and diet, genotype of the donor, and recipient embryo sex on GVH splenomegaly were marked ($p < 0.001$). $\underline{B}^{14}\underline{B}^{14}$ in every case were more competent than either $\underline{B}^{2}\underline{B}^{14}$ or $\underline{B}^{2}\underline{B}^{2}$. Conventional $\underline{B}^{2}\underline{B}^{14}$ and $\underline{B}^{2}\underline{B}^{2}$ donors on a conventional diet were again more competent than conventional on a tryptophane-deficient diet, which in turn were more competent than the gnotobiotic on the tryptophane-deficient diet. Donors were the same as for the CAMP assay indicated above. A total of 388 embryos were involved. Uninoculated embryos served as controls (Figure 13).

No parallel to GVH competence was found in the antibody response to the indigenous microbial flora as measured by the production of antibody to human blood groups of the ABH(O) system. Compare Figures 16, 17, 18, and 19, for instance, with Figure 11.

2. Antibody Against Human Blood Groups

Table 11 gives the overall acquisition of antibody to human blood group antigens of all animals in the gnotobiotic and conventional environments in terms of response or the lack of it. Figure 15 shows the percentage of responders histogrammatically. Although the percentage response in the $\underline{B}^{2}\underline{B}^{14}$ seemed greater overall in comparing conventional and gnotobiotic groups on tryptophane-deficient diet (since 100% of all conventional on conventional diet responded, no comparisons can be made here) this could not be demonstrated to be significant in doing an analysis of variance and applying the F-test. A modest environmental difference could be demonstrated only in the acquisition of anti-B ($0.025 < p < 0.05$) on the basis of percentage of individuals responding, although the tendency toward more frequent response in the conventional environment was definite. No gnotobiotic $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ or $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$ animals developed any antibody detectable through the use of A_1, Rh-negative cells as antigen following adsorption with O, Rh-positive cells. Only one $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$ developed a minimal response detectable at three months of age. One each of $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ and $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$ developed antibody detectable with B, Rh-negative cells, again detectable in the undiluted serum only at four months of age and at two months of age respectively. Two each of $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ and $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$ developed antibody detectable with human O, Rh-positive cells prior to adsorption, and six out of a total of fifteen $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$ responded.

With conventional animals on a tryptophane-deficient diet the two $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ animals which survived longer than one month did not develop antibody against A_1, Rh-negative cells. Two $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$ animals developed anti-A and one $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$ chicken did. Antibody against B, Rh-negative cells was developed by both $\underline{B}^{2}\underline{B}^{\underline{2}}$ chickens, five $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$ and seven $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$. Antibody detectable by human O, Rh-positive cells prior to adsorption was developed by both $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$'s, five $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$'s and seven $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$'s.

Figure 16 shows graphically the mean titres of antibody of all gnotobiotic animals to human O, Rh-positive cells (inasmuch as only a few of each genotype responded) and the mean titre acquired by the individual genotypes on a deficient diet in the conventional environment. It demonstrates that, given the stimulus, gnotobiotic animals can develop antibody, at least to the antigens used here, in titre equally as high as their conventional counterparts.

Figures 17, 18 and 19 show the acquisition of antibody to A_1 and B, Rh-negative cells and 0, Rh-positive cells by all three genotypes in the conventional environment on conventional diet. In no instance was antibody detected prior to two months of age in any of the individuals. In an attempt to determine whether or not the cumulative titres over a period of time were greater in any genotype as compared with others, ANOVA was performed on the total overall titres against the three blood group antigens, but significant differences could not be detected on application of the F-test. However, the tendency toward lower titres in the $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ as compared with the $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$ and $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$ is apparent from the graphs. Individual titres of responding animals for all groups are in Tables 12, 13, 14, 15 and 16. Again, the acquisition of antibody with a subsequent decline or complete loss could be seen in some, but not all of the responding animals during the course of the experiment. Moreover, it occurred at somewhat different times with different individuals although most individuals had acquired some antibody at four months of age in the conventional environment.

3. Weights of Animals

Animals were weighed periodically throughout the experimental period but particular stress was placed on the weight of the animals just prior to changing the diet to the tryptophane-deficient at 3½ weeks of age in the case of the gnotobiotic and their conventional counterparts, three months of age, and on the final weights of those animals living at the termination of the experiment at 200 or more days (Table 17). It had been noticed that a decrease in weight took place shortly after transfer to the deficient diet. However, this could not be shown to be significant on doing a paired analysis of pre- and post-transfer to the diet. Variation in genotype with environment occurred; $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$ being the heavier animals in the gnotobiotic environment and $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ being the heavier in the conventional. No genotypic differences existed at the time of termination of the experiment at which time the surviving conventional animals were heavier than the gnotobiotic.

Conventional animals on a conventional diet where weight gain was progressive confirmed differences in genotype. Table 17b gives the mean weights in grams at five to seven days, 26 days (age at which the other two groups were transferred to the deficient diet), at 102 to 109 days (just over three months of age) and just prior to termination (200+ days). With the exception of the first weighings, only females were included in the total mean since the two male $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$ and one male $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ were heavier than the females and their contribution to the overall mean weights of these two genotypes was not offset by males in the $\underline{B}^{14}\underline{B}^{14}$ group. Throughout, the $\underline{B}^{14}\underline{B}^{14}$ weighed less than either the $\underline{B}^{2}\underline{B}^{2}$ or $\underline{B}^{2}\underline{B}^{14}$'s. Moreover, paired analysis of weights at 26 days of age with animals (gnotobiotic and conventional) on autoclaved diet prior to change to tryptophane-deficient diet confirmed, as with the $\underline{B}^{2}\underline{B}^{2}$'s of the previous experiment, that autoclaving the diet had a marked detrimental effect on the growth of the chickens in spite of supplementation with vitamins. In some cases the mean weights of conventionally fed animals were as much as twice that of those on the autoclaved diet.

Survival or decreased longevity (Figure 20), does not under the experimental conditions nor the duration of the experiment confirm that the $\underline{B}^{14}\underline{B}^{14}$ genotypes are the least long-lived. The stress of the tryptophane-deficient diet seems to indicate an order of $\underline{B}^{2}\underline{B}^{2} < \underline{B}^{14}\underline{B}^{14} < \underline{B}^{2}\underline{B}^{14}$. Conventional on a conventional diet were not held long enough to determine differences in survival properly. It happened that two $\underline{B}^{2}\underline{B}^{14}$'s were the only deaths among these animals.

4. Antibody Production

The number of plaque-forming cells in the spleen representing antibody-forming cells after stimulation with sheep red blood cells were assayed by Fredericksen (see Ph.D. Thesis, T. Fredericksen, the University of Alberta).

E. Effect of Gnotobiosis on Immune Competency of Thymectomized Chickens

1. Graft-versus-Host Reactivity

The first assay for GVHR using thymectomized and non-thymectomized donors homozygous at the $\underline{B}^2$ histocompatibility locus was carried out at the time of transfer of animals from a corn-soybean initial diet to the tryptophane-deficient diet at age four weeks. The CAMP assay was employed. Recipient outbred embryos differing at the $\underline{B}$ histocompatibility locus from the donors were used. Embryo data were grouped according to surgical treatment and environment of the $\underline{B}^2\underline{B}^2$ donors (Table 18a).

Thymectomized animals in both environments were significantly less efficient donors ($p < 0.001$) while the gnotobiotic animals were less efficient as compared with the conventional ($p < 0.001$). Replicate analyses were also performed; these involved gnotobiotic animals only, two thymectomized and three sham operated (Table 18b). No significant difference could be demonstrated between replicate tests ($F_{1,118} = 0.3788$, N.S.) but a significant difference was confirmed between the number of CAM pocks in those embryos inoculated with cells from thymectomized as compared with non-thymectomized donors ($p < 0.001$); recipients of cells from thymectomized donors produced fewer CAM pocks.

These results are in contrast to those of Sheridan, Law and Ruth, 1969, in which GVHR competence as assayed by MES with the use of recipient embryos not differing at the

B histocompatibility locus showed that extirpation of the thymus in animals housed in conventional quarters actually increased the recipient embryo spleen weight. Moreover, Pazderka (personal communication) had confirmed the same through the use of outbred embryos with the same assay method. In order to re-examine this finding MES assays were, therefore, performed on both gnotobiotic and conventional chickens (as controls) using embryos differing at the B locus, as well as embryos carrying the same B histocompatibility genes, i.e., B^2B^2, prior to conventionalization of the gnotobiotes. The gnotobiotes were then transferred to quarters where adult White Leghorns had been housed on a constant basis, and where it was known that no particular precautions (other than the usual hygienic measures of cleaning and occasional fumigation between flocks) against transfer of any organisms had been taken, including that of Marek's disease virus (the causative agent of a horizontally transmitted lymphomatosis in chickens) which was known to have occurred previously in flocks housed in those quarters.

Mean $\log_{10}$ of MES with corresponding standard errors (Figure 21) is shown for types of recipient embryos pre- and post-conventionalization, as well as the corresponding uninoculated control embryos for a given group. Recipient embryos differing at the B histocompatibility locus of all groups including uninoculated control embryos showed a significant decrease in MES in the tests performed after conventionalization (in the case of the conventional animals already housed conventionally this refers to tests done at the same time as those originally housed in the isolators) as compared with those performed prior to conventionalization. The exception was the group of embryos inoculated with cells from thymectomized gnotobiotic chickens. In this, the only instance indicating any increase the F-test did not demonstrate that the increase was significant. However, the tendency toward an increase in the test embryos is remarkable inasmuch as it tends to lean toward the work of Sheridan, _et_ _al_., 1969. Nevertheless, at this time, three weeks after the exposure to the conventional environment, with the use of recipient embryos differing at the _B_ locus, the thymectomized donors produced a significantly lower GVHR as compared with the non-thymectomized in agreement with the results obtained by CAMP assay. Moreover, this was the case with animals housed conventionally throughout. It should, however, be noted that no known avian infectious disease, viral, bacterial or parasitic (endo- or ecto-) was known to have afflicted any animals previously housed in the quarters of the conventional controls (which were different quarters from those to which the gnotobiotic were transferred). At the same time, these animals, conventionally housed throughout, were still significantly more efficient donors, overall, than their originally gnotobiotic counterparts, confirming again, with regard to this aspect, the results obtained using the CAMP assay.

Where the recipient embryos were identical at the _B_ histocompatibility locus little difference existed in donor efficiency of gnotobiotic thymectomized and non-thymectomized either pre- or post-conventionalization although a tendency, not demonstrated to be significant, toward greater donor efficiency in the thymectomized existed. However, a pronounced and significant difference in MES in embryos inoculated with cells from thymectomized chickens housed conventionally throughout versus their non-thymectomized controls was demonstrated bearing out Sheridan's findings under experimental conditions duplicating hers. Although post-conventionalization tests of animals from the original gnotobiotic group showed no significant difference in thymectomized versus non-thymectomized, MES did increase in both groups significantly while, in this instance, uninoculated control embryos did not indicate a significant difference in the nature of the two groups of embryos employed. Moreover, since this was the last assay in the group of experiments, only one of the conventionalized, thymectomized chickens from the previous gnotobiotic group was living and the increase in this individual over the pre-conventional mean is the only one which can be compared. A comparison of this individual pre- and post-conventionalization showed an increase that was not significant. An increase in MES did not occur in the conventionally housed control animals.

All of the above results were independent of the number of circulating lymphocytes. The number of these was not altered by surgical treatment or environment (Table 19).

2. Antibody to Human Blood Groups

No thymectomized or non-thymectomized animals housed in the gnotobiotic environment originally acquired any antibody detectable through the use of A_1 and B, Rh-negative human red cells following adsorption with human O, Rh-positive red cells. Two thymectomized and three sham-operated (non-thymectomized) developed antibody to human O cells prior to adsorption, Table 20a. No antibody was detected before 2.5 months of age, and as previously, the presence of antibody was transient.

No antibody toward A_1, Rh-negative cells was acquired by any of the animals housed conventionally throughout. Only one thymectomized animal acquired antibody detectable with B, Rh-negative cells in the undiluted serum at 1.[illegible] months of age only. Two sham operated acquired anti-B detectable in both instances only once; in one case up to a 1:2 dilution at age two months, in the other in the undiluted serum only at age 1.5 months. Acquisition of anti-O (Table 20b) by conventionally housed animals (one out of five thymectomized, and four out of six sham-operated) was detected first at 1.5 months of age and the same transience noted previously was observed here, as well.

3. Growth and Weight

Two separate hatches comprised the animals used in this experiment. The first involved gnotobiotic animals only and the second hatch included the conventional controls. Weights were taken at different times as indicated in Table 21. The weights in grams were converted to $\log_{10}$ for analysis of variance and subsequent F-testing. In no instance was there a significant difference in weight between thymectomized chickens and sham-operated (Figure 22). Neither was there a significant difference in weight gain or loss over the period of time in which weights were taken, namely, the earliest being at 39 days of age (ten days after the change to a tryptophane-deficient diet) and the latest was 92 days of age. With the second hatch where, at 78 days of age, the gnotobiotic weighed less than the conventional ($0.01 < p < 0.025$). Moreover, in calculating the overall mean weights of the gnotobiotic versus the overall weights of the conventional for all the occasions in which weights were taken, the gnotobiotic were still significantly lower.

4. Survival

Five animals in total, out of the groups used here died within 24 hours after surgery. Of those surviving the surgery, the thymectomized groups in both environments experienced the greatest numbers of deaths, Figure 23 (also Table 21).

F. Effect of Gnotobiosis on the Immune Response of Bursectomized Chickens

1. Graft-versus-Host Reactivity

The effect of gnotobiosis on the GVH competence of chickens with the bursa of Fabricius removed was examined, as previously, by comparison with conventionally raised controls. Since surgical procedures in germfree facilities tend to be cumbersome as is the case with x-irradiation of germ-free animals (total body irradiation following bursectomy has been deemed necessary to eliminate cells that maintain the germinal center and plasma cell line of differentiation, van Meter, Good, and Cooper, 1969) it seemed reasonable to evaluate a method of chemical bursectomy which was not reported to interfere with other lymphoid organs. In 1970, Lerman and Weidanz reported that antibody formation and immunoglobulin synthesis were severely depressed when measured in adolescent chickens treated at hatching with cyclophosphamide. Linna, Frommel and Good (1972) reported treatment with cyclophosphamide caused an initial absence of bursal lymphoid cells with subsequent destruction of bursal architecture and deficiency in immunoglobulins. Moreover, in light of seemingly encouraging reports on the ontogeny of bursal function in the chicken through the use of this method of bursectomy by Toivanen, _et al_. (1972 and 1973) it was included in the experimental protocol for evaluation and comparison with surgical bursectomy. Both surgically bursectomized and sham operated controls together with cyclophosphamide treated animals in both environments were used. The more sensitive of the GVH assays, the CAM pock test was employed. Recipient embryos were outbred, of unknown B genotypes; the donors were inbred $\underline{B}^{\underline{2}}$ homozygotes.

Embryo data were grouped according to surgical or chemical treatment, and microbial status (environment) of the donor and subjected to analysis of variance as before. No significant difference was detected between any of the treatments, surgical bursectomy, surgical bursectomy plus cyclophosphamide-treated, sham-operated and sham-operated plus cyclophosphamide treated in either the germ-free or the conventional environment (Figure 24). Donors included: one bursectomized, five bursectomized and cyclophosphamide treated, three sham operated, and five sham operated and cyclophosphamide treated from the germfree environment; three bursectomized, three bursectomized and cyclophosphamide treated, four sham operated and seven sham operated and cyclophosphamide treated from the conventional environment. All isolator animals were germfree at the time of analysis. Included in the germfree group was one animal, a late hatch which was treated in no way whatsoever, not even subjected to the anaesthetic or preliminary surgical trauma as were the sham operated. It likewise was not significantly different from any of the other groups. The mean for all embryos did not differ appreciably from the mean obtained by grouping embryos according to individual donors and averaging the donor means (Figure 24b). Under general conditions of physiological well-being the GVHR to major histocompatibility (B) antigens is not influenced by cells of bursal derivation and this, in turn is not influenced greatly by the presence or absence of micro-organisms harboured by the host. The overall mean $\log_{10}$ for the gnotobiotic was slightly lower than that for the conventional (0.9737 versus 1.0680). Converted to arithmetic values this is 9.19 as compared with 11.69 representing the number of pock producing cells per 0.1 ml. of donor whole blood.

In addition to determining the influence of an indigenous population of micro-organisms on infectious hypersensitivity, an attempt was made to determine whether the GVHR could be influenced by intentional induction of hypersensitivity. GVHR was assayed using CAMP prior to and immediately following skin testing on two separate occasions; once before and after skin testing with P.P.D. (purified protein derivative) three weeks after injection of BCG vaccine, and secondly before and after skin testing with crude BCG after allowing an additional eight weeks to pass with the hope of increased hypersensitivity. Just prior to the first skin test the animals had become monocontaminated with Staphylococcus epidermidis.

The first test, Figure 25, produced bizarre and uninterpretable results as follows: (1) a significant depression of GVHR occurred amongst the gnotobiotic sham operated regardless of any previous exposure to Mycobacteria after skin testing whereas the bursectomized were unaffected; (2) sham operated gnotobiotes were also more competent than the bursectomized, at this point in time; (3) all bursectomized conventional animals were significantly depressed in GVH competence following skin testing, again regardless of exposure to BCG vaccine, whereas the sham-operated showed variability with no specific pattern and no significant overall difference; (4) sham operated conventional chickens were significantly more competent than were their conventional bursectomized counterparts; (5) unexpectedly, the gnotobiotic animals were significantly more competent overall than were the conventional ($0.001<p<0.005$).

On direct inspection it could not be seen that skin thickness measurements differed in those animals having been vaccinated with BCG although later analysis of variance did show that conventional animals exposed to BCG did react significantly in comparison with the non-vaccinated. No gnotobiotic animals, regardless of treatment, could be shown to be hypersensitive to Mycobacteria as measured by skin thickness (Table 22). Since differences were not obviously apparent, with the additional bizarre effects on GVHR, and since hypersensitivity to tuberculin is reputedly difficult to elicit in the avian species the skin testing was repeated (Table 23) a number of weeks later. This allowed more time for sensitivity to develop. Repeat GVHR (Figure 26) showed no difference pre- and post-skin testing in either environments or between any of the treatment groups.

Insofar as one isolator containing four animals had become contaminated with a fungus in the interim, these were not included in the repeat skin tests to avoid the possibility of cross reactivity of Mycobacteria with fungal products capable of eliciting hypersensitivity, thereby obtaining false positive reactions. None of the gnotobiotic animals could be shown to be hypersensitive.

It is concluded, therefore, that delayed type hypersensitivity to an infectious agent does not influence GVHR. Any proliferative activity of lymphoid cells invoked by the hypersensitive state is independent of GVH competence as measured by the CAM pock test. No antigenic specificities seem to be shared and any influence by a viable Mycobacterium (BCG in this case) is not transferrable to those immune activities or cells involved in the CAM pock test.

No significant difference in GVH competence between gnotobiotic and conventionally housed chickens could be demonstrated although the overall mean of the gnotobiotic was, as in previous experiments, slightly lower.

2. Testing for Hypersensitivity

Table 22a gives the skin thicknesses in inches, as measured with an engineer's micrometer of the right and left wings webs of chickens following injection of P.P.D. intracutaneously into the web of the right wing of gnotobiotic animals. Table 22b gives the same for conventional animals. Analysis of variance and F-testing were done following conversion to $\log_{10}$ of the web thickness x 10^3. Right wing thickness compared with left wing thicknesses were increased immediately following injection presumably due to irritation following the injection and in some instances where feathers were plucked away small petichiae appeared. However, with increased experience, the irritation could be reduced although not eliminated such that significant differences between thickness of right and left wing web disappeared by four hours as compared with eight hours in the initial attempts. In no instance were the injected webs significantly greater in thickness than the uninjected webs (i.e., right webs versus left webs) in gnotobiotic animals, from 24 to 72 hours, the time at which delayed hypersensitivity is generally considered to show its peak reaction on stimulation (Table 24). Neither were injected web thicknesses of those animals having received BCG vaccine greater than injected web thicknesses of animals not having received BCG. This latter was not the case with conventionally housed animals, where, after the initial decrease in injected web thickness a gradual increase occurred such that at 8, 24, 48 and 72 hours a significant difference between right and left webs was apparent. The right webs (injected) of those chickens having received BCG vaccine were significantly thicker than were those of animals not having been previously vaccinated at 24, 48 and 72 hours, conforming in time, at least, to the classical definition of reactivity in delayed infectious hypersensitivity. The same held true in the instance of repeat skin testing with crude BCG (Table 23 and Table 24) although the time of significant response was at 48 and 72 hours. At 72 hours injected right wing webs of gnotobiotic chickens were also greater than the uninjected left webs, but this could not be demonstrated to involve BCG vaccinated only. The general overall response for both skin test series is in Table 24.

In no instance were there necrotic or hemorrhagic areas noticeable (besides the initial irritation described above) and no central "wheal" existed in conventional animals where BCG vaccinated animals exhibited increased skin thicknesses. No histological examinations of the site of skin testing were done. Anderson, 1971, did histological work on the skin test area of wing webs of chickens and concluded that the infiltration of mononuclear cells, seemingly of lymphoid origin, corresponds to the classical description of delayed hypersensitivity. However, this work differs in that gnotobiotic chickens could not be shown to be sensitive as compared with Anderson's very young germfree fowl (injected at seven days of age with heat-killed Mycobacterium avium and tuberculin tested three weeks later). It is possible that dosage differences, Mycobacterial strain and timing of skin testing could be involved. The results are, however, in keeping with those of Sasaki (1974) who could not elicit delayed type hypersensitivity in germfree mice without mono- or di-association with certain other organisms. This suggests a possible adjuvant effect of the indigenous microbial flora.

3. Capillary and Ouchterlony Precipitation Tests

In no instance could circulating antibody to tuberculin products be demonstrated with these two tests under the conditions described in Materials and Methods. Final tests with plasmas obtained at the terminal bleeding were also negative. It is, therefore, doubtful that any positive reactions in skin-testing could be ascribed in any measureable extent to humoral antibody of an Arthus reaction within the limitations of either test.

4. Cunningham Plaque Assay for Antibody Producing Cells

This particular test for the number of cells in the spleen capable of producing antibody on primary stimulation was intended as a measure of the effectiveness of both methods of bursectomy but in particular for the effectiveness of the chemical bursectomy with cyclophosphamide. Animals had been given a standard dose of sheep red blood cells five days prior to termination at which time the spleen was removed and the cells from it tested for the production of antibody against SRBC. Table 25 gives the number of plaque forming cells per 10^6 spleen cells in duplicate in the order of treatment of the animals. No overall difference was noted in duplicate enumeration of plaque forming cells. Moreover, although there was a tendency for fewer plaque forming cells in spleens of animals from the gnotobiotic environment, this could not be demonstrated to be significant. Surgical bursectomy markedly decreased and in some instances completely obliterated the production of plaque forming cells (antibody producing cells) to SRBC on primary stimulation.

The effect of cyclophosphamide in this particular experiment cannot be considered as eliminating cells of bursal origin in the dosage and conditions of the experiment. In the gnotobiotic and conventional environments opposite effects of cyclophosphamide appeared to have taken place, namely, enhancement of the number of plaque forming cells in animals from the gnotobiotic environment (i.e., comparing sham operated and cyclophosphamide treated only) and a depression thereof in the conventional environment. In neither environment was there any instance of as dramatic an elimination of antibody producing cells as with surgical bursectomy.

A direct correlation (calculated according to Snedecor, (5th ed., 1956) between body weight and spleen weight (Figure 27a and b) in the gnotobiotic chickens suggests that the weight of the spleen in this instance is influenced primarily by body weight whereas other variables are at play with the conventional animals where no correlation existed. Table 26 gives grams of body weight and milligrams of spleen weight. These were changed to $\log_{10}$ for the test of correlation and the analysis of variance. F-tests indicated that both spleen weight, as well as body weight were significantly higher in the gnotobiotic environment.

The portion of spleen removed for histological observation did not indicate any differences morphologically between environment or treatment groups except that spleens from sham operated animals showed foci of mononuclear cells (possibly lymphoidal) which were absent in the bursectomized animals. Presumably because of the tryptophane-deficient diet these chicken spleens did not exhibit the dense, convoluted architecture of the usual chicken spleen (R.F. Ruth, personal communication) but rather appeared much closer to the text book descriptions of embryonic spleens.
Figures 28a and b show representative sections from bursectomized and sham operated animals respectively, illustrating, in the latter case, the accumulation in a single focus of mononuclear cells. These were considered to be the secondary foci referred to by Thorbecke, _et al_., 1957, and by Pierce, Chubb and Long, 1966.

5. Titration of Plasmas for Determination of Antibody Against Sheep Red Blood Cells

None of the bursectomized animals in either the gnotobiotic or conventional environment had any detectable antibody to sheep red cells prior to an injection with the same, and developed none in the five days following. Of the gnotobiotic non-bursectomized (sham operated) animals only the one totally untreated animal had any antibody to sheep red cells (titre = 1/4) prior to SRBC injection. Five days later all had developed antibody to a titre of 1/64 or higher. Amongst the conventional sham operated an increase in antibody to 1/32 or higher occurred. Again, only one animal showed any antibody against sheep red cells prior to the injection and that only in the undiluted serum (Table 27).

6. Acquisition of Antibody Against Human Blood Groups

With one exception, no gnotobiotic animals, irrespective of treatment developed antibody detectable with A_1, Rh-negative human red cells following adsorption with O, Rh-positive cells at any time. The exception, a sham operated animal, developed antibody detectable in a 1/4 dilution of plasma five days following injection of sheep red cells.

Only sham operated gnotobiotic animals developed antibody detectable with B, Rh-negative cells for the first time at age five months. Two out of the seven surviving at this time had antibody in the undiluted or 1/2 dilution of plasma prior to injection of sheep red cells. Five out of the seven developed anti-B in the five days following injection of SRBC ranging in titre from 1/2 to 1/8.

Figure 29 shows histogrammatically the acquisition of anti-A_1 and anti-B by sham operated, gnotobiotic animals prior to and following the injection of SRBC. Figure 30 compares the acquisition of antibody detectable with human O, Rh-positive cells prior to adsorption with O cells, as well as antibody to SRBC pre- and post-injection of the same.

Three bursectomized gnotobiotic chickens had antibody detectable with human O, Rh-positive cells only once in the undiluted serum. All other bursectomized animals (with the exception of two conventional bursectomized) acquired no antibody whatsoever against human blood groups. Tables 28, 29, 30 and 31 give the acquisition of anti-O by gnotobiotic animals, and anti-A_1, anti-B and anti-O plus any species determinants by conventional sham operated animals (and one bursectomized which developed anti-O detectable in the undiluted serum once). Figure 31 and 32 represent the increase in anti-A_1 and anti-B, Rh-negative in conventional sham operated animals pre- and post-injection of SRBC, and the acquisition of anti-O and anti-SRBC at the corresponding time.

7. Growth and Survival

Animals were weighed once only at the termination of the experiment at which time body weights were correlated with spleen weights (Table 26). Bursectomized gnotobiotic animals survived less well than the gnotobiotic non-bursectomized (Figure 33) whereas percentage of survival was nearly equal in the conventional environment. This refers to surgically bursectomized animals only, inasmuch as only one conventional non-bursectomized, cyclophosphamide treated animal died.

DISCUSSION

A. Housing of Animals

1. Isolation Units

In most of the experiments performed here, the isolator animals must be termed gnotobiotic insofar as the microorganisms with which they were contaminated were defined as indicated in Tables 1M, 2M, 3M, and 4M in the Appendix. Experiment 1 (Table 1M) refers to the initial experiment for the effect of gnotobiosis on the natural immune response employing $\underline{B}^{2}\underline{B}^{2}$ gnotobiotic and conventional chickens, Experiment 2 (Table 2M) to the experiment involving $\underline{B}^{2}\underline{B}^{2}$, $\underline{B}^{2}\underline{B}^{14}$, $\underline{B}^{14}\underline{B}^{14}$ genotypes, Experiment 3 (Table 3M) to the thymectomy experiment and Experiment 4 (Table 4M) to the bursectomy experiment. The term SPF (for "specific pathogen free") is avoided since the term implies only a more hygienic environment in which no known pathogens are present and in which there may be a reduction in the variety of microorganisms inhabiting the experimental animal but these are not defined. The bursectomy experiment is the only one in which the term "germfree" would apply in the initial stages. Variations in significant differences in donor competence of gnotobiotic and conventionally housed animals in GVHR could, in part, be attributed to the nature of the contamination or the extent of it at any given time.

2. Preparation of Eggs for Hatching

The procedure finally adopted for the preparation of embryonated eggs (Materials and Methods) proved remarkably effective in bringing eggs into the isolation chambers sterilely. However, hatchability was reduced similar to the findings of Harrison, 1969. Early experience in spraying eggs with peracetic acid was disappointing from the point of view of obtaining germfree hatchlings and was not attempted again although others have reported satisfactory results with improved hatchability (Jol *et al*., 1972; Harrison, 1972).

3. Preparation of Feed

In all except the final bursectomy experiment, the feed sterilized by autoclaving as indicated in Materials and Methods proved to be inadequately sterilized sooner or later in the course of the experiments. Sporulating bacteria, primarily Clostridia and Bacillus species, as well as fungi recovered from the animals were also recoverable from both the autoclaved and unautoclaved feed. The fear of possible production of toxic materials through the use of gamma irradiation for sterilization seemed very much unfounded after the reports of Coates, Ford, Gregory and Thompson, 1969; Ley, Bleby, Coates and Paterson, 1969; Porter and Festing, 1970; and Eriksen and Emborg, 1972. The lack of weight gain of $\underline{B}^{2}\underline{B}^{2}$ conventional animals and of the three genotypes, $\underline{B}^{2}\underline{B}^{2}$, $\underline{B}^{2}\underline{B}^{14}$, $\underline{B}^{14}\underline{B}^{14}$, prior to the changeover to a tryptophane-deficient diet were additional contraindications for the further use of the method of autoclaving. Coates and her associates analyzed diets for loss of vitamins at doses of gamma irradiation ranging from two to five Mrads and reported losses which were much less than those given by Luckey, 1963, for heat sterilization (autoclaving). Moreover, the effect of irradiation on proteins, amino acids and fats in dosage as high as seven Mrads was negligible. No difficulty in regards to palatability was encountered. Subsequent storage presented no problems. Coates' quantitative studies on inactivation of microbes in diets were remarkably thorough and impressive.

Pre-implantation deaths of mouse eggs in mice fed irradiated diets have been reported by Moutschen-Dahmen et al., 1970, but the same could not be confirmed in rats by Eriksen and Emborg, 1972. Porter and Festing reported that the effects of diet on breeding mice resided more in the nature of the diet than in the method of sterilization whereas palatability testing indicated a strong preference for irradiated diet over autoclaved diet.

In our experience gamma irradiation as a method of sterilization reduced the hazard of contamination to that of human error. The bursectomy experiment was the most successful in maintaining animals in the germfree state; two animals remaining germfree for nearly six months at which time all experimental animals were deliberately terminated.

Five chickens of the English Game Hen variety kept for purposes of potential breeding and serological work were maintained in the germfree state according to the cultural criteria in Materials and Methods for eight and one-half months.

4. Monitoring for Sterility

Although the schedule of culturing in the types of media outlined covered the possibilities of the majority of contaminating organisms most likely to be encountered such as aerobic and anaerobic gram-positive cocci and bacilli, gram-negative aerobic and anaerobic organisms, and fungi, the possibility of Mycoplasma cannot be excluded. Inasmuch as, Mycoplasma can be transmitted vertically (i.e., from the hen *in ovo* to the developing embryo) a more thorough delineation of the microbial status of the embryonated eggs would have been to have sample embryos specifically cultured for P.P.L.O. and *Mycoplasma gallisepticum* in particular, at one of the veterinary laboratories in the province.

No necropsied animals showed lesions of liver, spleen, heart, lungs or intestines on gross examination, which would have led to any suspicions of viral diseases. Except for the fact that the viscera of the necessarily limited numbers of chickens maintained in isolation facilities were used for bacterial cultural purposes, submission of samplings of some isolator animals might have assisted in confirming the absence of infections of viral etiology. It is, nevertheless, reasonable to assume, that since the filtration system on isolation units was efficient in filtering out bacteria (since all bacterial and fungal contamination could be traced to either the feed or to human error) that any viruses transmissable on particles (i.e., horizontally), at least to the size of 0.5 mu, most likely were absent. Hence, a reduction in viral population in the isolators, even though not designed to exclude viruses is a strong likelihood.

B. Effect of Gnotobiosis on Natural Immune Competence

1. Graft-versus-Host Reactivity

The extent of differences in GVHR between gnotobiotic and conventional animals varied. However, the gnotobiotic were consistently less competent as donors in the GVH reaction as measured by the CAM pock test and by weighing spleens of outbred embryos after intravenous injection of peripheral blood. Although, in some instances (e.g., three months of age when measured by CAMP assay and four months of age as measured by MES) differences could not be demonstrated to be significant statistically, the gnotobiotic animals never reached the level of competence of the conventionally housed. Possible factors involved in the extent of the differences could be the source of embryonated eggs used to produce the germfree animals. This would seem to have been circumvented as much as possible since all $\underline{B}^2\underline{B}^2$ homozygous chickens used were from eggs purchased from Hyline Poultry Farms. Therefore, it would be anticipated that within a group, at least, they would react consistently. Further to this, one could invoke the genetic makeup of the donor as regards fine structure of the gene(s) at the $\underline{B}^2$ blood group and histocompatibility locus and/or seasonal variations within a genetic strain. However, both of these factors are without experimental support as carried out here. Inasmuch as, recipient embryos were outbred and of unknown genetic constitution similar arguments could apply to the reaction provoked by their antigens. Similar variation was observed with the three genotypes, $B^{2}B^{2}$, $B^{2}B^{14}$, and $B^{14}B^{14}$ in the experiment following and is discussed in more detail there.

The establishment of an increased reaction in GVH splenomegaly in recipient female embryos as compared with recipient male embryos is a significant one. The simplest, most direct interpretation involves the reaction of donor cells to an antigenic component, the synthesis of which is genetically controlled by a gene (or genes) on the W chromosome of the female not present in the male (in the avian species ZW is the female; ZZ is the male).

A second interpretation could involve the more rapid maturation of cell surface structures including blood group and histocompatibility antigens in the female. This invokes a possible hormonal control associated with the sex chromosome (W chromosome). Hence, if female embryos are more advanced in the development of cellular antigenic constituents, the introduction of donor immunocompetent cells would result in increased reactivity of these cells against host antigens. However, this interpretation (as well as the first, above) should be amenable to testing by the other assay for GVH competence, the CAM pock test. If maturation of host cell surface receptors or a distinct antigen is involved a similar increase in GVH should be demonstrable in the number of pocks on the CAM from recipient female embryos. Although the sex of recipient female embryos was not recorded for CAMP assays, attempts by others (R.F. Ruth and B.M. Longenecker, personal communication) have consistently failed to demonstrate any differences between male and female recipient embryos with this assay method.

This leads to a modification of the second interpretation, above. A hormonal control associated with the sex chromosome phenotypically expressed as increased immunological maturity or competence in the female may be the explanation for heightened MES. The embryos were incubated for eight days following the injection of donor whole blood. If the female embryo were sufficiently mature to mount an immune response against donor cells the eight days would be enough time to measure this. The final MES may be the result of combined GVHR and host against graft reaction.

2. Antibody to Human Blood Groups

Several aspects of the work reported here extend the work of others on the acquisition of antibody without deliberate and specific stimulation. Some specific points on the nature of the immune response are brought out in relation to the interpretation of humoral antibody production under both natural and experimental conditions.

Although Springer has reported that germfree chickens acquired antibody adsorbable with human O, Rh-positive red cells at the approximate age of 30 days on an "antigen-free" diet, this did not occur at all in this particular group of animals. This may be due partially to the tryptophane-deficient diet and partially from the fact that most of the organisms cultivated from the feed (the primary source of contamination) were gram-positive, not known to invoke antibody formation against human blood groups. However, even conventional animals on a conventional diet did not develop antibody until two months of age.

All conventional animals on the tryptophane-deficient diet acquired definitely lower titres than did those conventional animals on a conventional diet. Several explanations can be offered. Firstly, since tryptophane is an essential amino acid for the chicken as it is for man, the synthesis of new materials including the gamma globulins of antibodies could be restricted to that synthesized by extraction of essential amino acids from and at the expense of other body cells and tissues. This then provokes the question as to why anti-A_1, anti-B and anti-O were not limited to the same amount either by titre or in all individuals. Secondly, since weight gain was reduced immediately on the autoclaved diet relative to the unsterilized diet, one can assume that in general a destruction of many essential growth factors occurred. Antigenic materials must also have been denatured thereby decreasing the stimulation of lymphoid tissue in the intestine. Thirdly, it is conceivable that a viable ingested population of microorganisms have a greater stimulatory effect in antibody production on a continuous basis than do killed organisms. Hence, those bacteria or fungi carrying substances identical or similar to blood group substances, may have contributed little in antigenicity in the autoclaved diet.

Springer, 1962, has pointed out that chickens carry antigens on their own cells and tissues similar to human blood group A. Hence, one would expect that an animal would not produce antibody to substances of which its own cells are composed. No attempt was made here to titrate with A_2 cells, for instance, in addition to A_1 cells so that production of antibody to various modifications of the A substance was not measured. However, the fact that all conventional animals on a conventional diet did acquire some antibody against A_1 cells infers that this A substance is somewhat different from that carried on the animal's own cells (if indeed, this is the case), or, that the particular strain of chickens used here do not, in fact carry a substance similar to the human A_1 substance as we know it. One is inclined toward the first of the two possible explanations on consideration of the fact that much less anti-A_1 was acquired when it was acquired, than either anti-B or anti-O. The antibody could be considered as having been produced toward that portion of the molecule representing A substance which is modified or different from the structural architecture of the A molecule on the chicken's own cells which represents the receptor site for the antibody. This could involve either a chemical or configurational difference in a portion of the molecule.

None of the animals on a tryptophane-deficient diet acquired antibody detectable with human A_1 red cells. Several interpretations could be involved: (a) if tryptophane is an integral part of the antibody molecule against A_1 its unavailability prevented the synthesis of the molecule; (b) since the animals were on a sterilized diet (by autoclaving) throughout, those viable microorganisms involved in stimulating the production of this antibody may have been absent; (c) substances similar to A in the diet proper could have been denatured by heat.

The observation that antibody toward human blood groups was acquired, but disappeared sometime after four months, then reappeared poses an interesting series of questions. The period of time of diminishing antibody, approximately four months after hatching corresponds roughly to the time of regression of the Bursa of Fabricius. Antibody reappeared, or increased in titre sometime later but exactly when, or for how long the period of decreased or no antibody to human blood groups lasted was not determined in these or some of the later experiments. From later work with antibody against human blood groups in older adult chickens the suspicion was implanted that the character of antibody might be somewhat different post remission of antibody than that prior to the remission. The antibody reacting with human O, Rh-positive cells and with B, Rh-negative cells was nearly as high in titre prior to remission as post remission. However, the number of adsorptions required to eliminate antibody reacting with O cells were doubled later implying that either there was more antibody against O cells of a heterogeneous nature which could be eliminated only with repeated adsorptions, or that an increase in antibody against species determinants not definable within the human ABH(O) blood group system occurred. One is, however, confronted with the problem of the derivation of antibody at two different times when there is, for all intents and purposes, a continuous stimulation.

Wong, 1974, found that allofixing antibody of chickens is primarily IgG but that some of the allofixing antibody of primary antisera was sensitive to mercaptoethanol indicating that some of the antibody may be IgM. If one accepts the premise that only a cell committed to the synthesis of an endproduct (be it myosin, hemoglobin, chondroitin or whatever) in a qualitative sense, i.e., "all or none" can synthesize that endproduct, the above findings imply that different cell populations from which different endproducts are derived in the form of different immunoglobulins may be involved. That the endproduct of one cell line, possibly bursal in origin, could be represented by the original antibody to A, B, and O with the remission representing an interim during which potentially immunocompetent cells from whatever source are differentiating, maturing and/or proliferating to take on the function of the original cells seems a reasonable conjecture. The second rise in antibody to A, B, and O would then represent the end product of a second line(s) of cells. No attempt was made to determine the immunoglobulin spectrum of the antibody formed. This does, however, present an area of further investigation with the potential of contributing more detail on the differentiation and kinetics of populations of immunocompetent cells in the avian species. Insofar as, some antibody in very young chickens, presumably of maternal origin, and presumably IgG, was found, the concept of heterogeneous antibody is strengthened. Antibody against human blood groups is, by and large, considered to be IgM, i.e., of high molecular weight. One might infer that the mother hen possessed more than one type of antibody to a given antigen; that of smaller molecular weight was passed on to the young.

Moreover, one can invoke seasonal variations in immune responsiveness, a phenomenon requiring a greater length of time for study than that used here. However, it is established in this and in the following experiments that the base level of "natural" antibody in a given animal is not at the same level at all ages. Therefore, any stimulation with cross-reacting antigens could result in a markedly variable response.

3. Growth and Weight

Growth and weight as described in Results indicate a pronounced detrimental effect of autoclaving the diet. Sasaki, 1970, has reported poor growth with overzealous sterilization of the diet in mice and has suggested that some of the reports on poor survival of mice upon conventionalization might have mistakenly attributed this to a depressed immune system rather than to a nutritional source. It is quite possible that in those instances documented here where immune response was depressed that the effects were secondary to the diet.

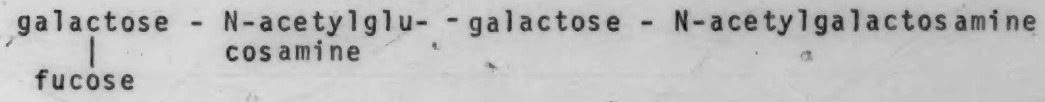
4. Survival

The nature of the diet given the chickens here of necessity placed limitations on survival and secondarily the duration of the experiment. It would seem that the gnotobiotic animals, although housed together in threes did not pick at one another and cannabalize each other to the same extent as the equivalent conventional animals on a tryptophane-deficient diet. Deaths by cannabalism amongst the gnotobiotic were more or less non-existent. Although weights of the animals were similar, it is adjudged worthy of mention that the feathering of the gnotobiotic animals was better, and the general appearance of well-being seemed superior.

C. Determination of Antibodies to Human Blood Groups in Adult White Leghorn Chickens

During the time that plasmas were collected for titrations from gnotobiotic and conventional chickens a variety of tests on chickens available in the Biosciences Animal Center were also performed in an attempt to characterize the system more fully.

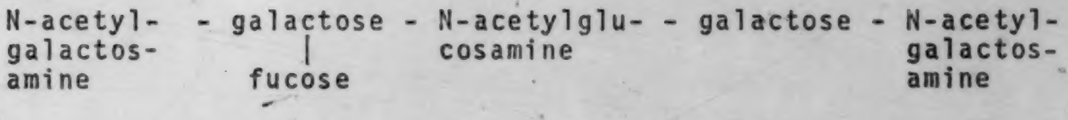
On the premise that individual "species" antibodies are formed as well as antibodies to specific blood groups or the receptor sites on cell surfaces designated as blood groups, the totality of these antibodies to human cells should be detectable with H(O) cells (in clinical terms simply designated as O cells with an Rh-negative or positive designation). Further, if the serum or plasma antibody to H-substance characterized chemically as



$$\underset{\text{fucose}}{\underset{|}{\text{galactose}}} - \text{N-acetylglucosamine} - \text{galactose} - \text{N-acetylgalactosamine}$$

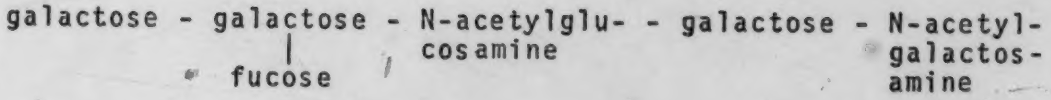
(see Race and Sanger, 1970), should be removed by reaction with the substance proper or with cells typed as H(O) as is done in adsorption, it should be possible to detect antibody to A or B blood groups remaining in the serum or plasma. These substances contain N-acetylgalactoseamine and galactose, respectively, added to the above structure thus:

(A)



$$\text{N-acetylgalactosamine} - \underset{\text{fucose}}{\underset{|}{\text{galactose}}} - \text{N-acetylglucosamine} - \text{galactose} - \text{N-acetylgalactosamine}$$

(B)



$$\text{galactose} - \underset{\text{fucose}}{\underset{|}{\text{galactose}}} - \text{N-acetylglucosamine} - \text{galactose} - \text{N-acetylgalactosamine}$$

Consequently, antibody against these two substances can then be titrated separately.

It can be argued that adsorption with O cells could result in the loss of some antibody against A and B as well as other antibody. Nonetheless, in the absence of large quantities of pure blood group substances the method used here constitutes "next best" choice.

In contrast to the observations with the younger conventional chickens described above, the differences in antibody against A_1 and B, Rh-negative cells were much less pronounced in older, adult chickens to the extent where statistical analysis of numbers of these did not do more than indicate a tendency toward greater antibody titre to B.

Since the plasmas were taken from chickens of an age where bursal regression should have been complete, this lends supportive evidence to the concept that: (a) multiple immunizations (as one would anticipate occurs from external microbial and dietetic stimuli) are not necessarily dependent on bursal presence or intactness, and (b) that different cell populations or lines might be involved. Moreover, most of Springer's work on the acquisition of antibody by chickens against human blood groups has been done on young chickens (whose blood groups and/or histocompatability type he did not define) at the age where early antibody is acquired.

Consequently, his assertion that chickens do not acquire antibody against human A blood group substance because of similar structures on their own cells may be a concept requiring some modifications, most conspicuous of which would involve the concept of heterogeneity of antibody produced as discussed for the previous experiment.

Of more pronounced interest is the observation that removal of the right (or both right and left) superior cervical sympathetic ganglion in male chickens on the day of hatching depressed the ability of these to acquire antibody detectable in the unadsorbed plasma with A_1, and B, Rh-negative and O, Rh-positive human red cells. Removal of the left ganglion showed a depression, as well, but not so pronounced. Because the differences lay in the unadsorbed plasma with the three cell types it could not be considered specific for A or B human blood groups nor necessarily to blood group O insofar as one would expect antibody to other surface receptors not necessarily definable within the ABH(O) system to be detected under these circumstances, as well. Nonetheless, it constitutes a further observation to those made by Alsager (1969) wherein the removal of the superior cervical sympathetic ganglion released the male thymus from an undefined inhibition in response to thyroxin (measured by increase in weight of thymus), which in turn elicited a greater response in the female. However, in what way these observations are related is difficult to interpret. Since, from Alsager's work, one would infer that ganglionectomy should enhance immunological responsiveness, at least in those aspects of thymus dependent responsiveness in the male, the decrease in a response, namely, humoral antibody production, dependent presumably on bursal function seems a strange inverse effect. Alsager did not directly test thymic function; only an increase in weight was measured. He also did not test the effects on the bursa, either functionally or grossly. One could argue, additionally, that although humoral antibody response is considered to be a parameter of bursal cell differentiation, development and/or proliferation, and that this same response following the regression of the bursa is due to previous seeding of precursor cells to other lymphoid organs this concept may be overly simplistic (Edwards, _et al_., 1968). It is not unreasonable to think that certain thymic cells could differentiate to become antibody producing cells in the absence of bursal cells following bursal regression. The animals used here were well past the age of bursal regression and the effect of the ganglionectomy may have been exerted in the long range on a cell line responsible for antibody production but derived from the thymus. The acquisition of antibody to human blood groups was depressed on deliberate extirpation of the thymus (see Results: Effect of Gnotobiosis on the Immune Competence of Thymectomized Chickens). Similar to Alsager's assessment, one cannot say here that the decrease in acquisition of antibody against human red cells in the male is dependent on the presence of the ganglion or on the intactness of the sympathetic chain. Why the effect should lean toward the unilateral is difficult to determine and why the progressive difference in reactivity of male unadsorbed plasma to $A_1 < B < O$ cannot be resolved within the limitations of the experiment. The implications are simply that the antibody may be heterogeneous in nature and may involve a cell surface receptor site not involving the ABH(O) blood group system *per se*, but whose cross-reactivity with this major human blood group system is less by virtue of the sympathectomy in combination with androgen effects in the male. Sheridan, 1967, has reviewed the literature on the effect of androgens on the lymphoid system of the avian species.

D. Effect of Gnotobiosis on Immune Competence of White Leghorn Chickens of Different Genotype Histocompatability Antigens

1. Graft-versus-Host Reactivity.

Significant differences in CAMP between gnotobiotic and conventional donor animals did not exist until six months of age at which time MES (performed only at this time) also indicated the same difference in donor competency, the gnotobiotic being less competent. It could be argued that the later differences may have had their original source in the tryptophane-deficient diet and secondarily through subsequent association with a microbial population as the animals became contaminated. None of the contaminating organisms were known to be pathogenic in the status of their association and there was no evidence of overt disease among any of the animals. Nevertheless, they could conceivably have been a source of drainage on the resources of the host. Wostmann, Pleasants and Bealmear, 1969, obtained evidence that numbers of leukocytes (both lymphocytic and granulocytic) in CFW germfree and conventional mice were affected far more by diet than by microbial status. Pathogenicity of Salmonella typhimurium in germfree rats upon monoassociation with this organism could be greatly influenced by the type of diet fed the rats, all of which were seemingly healthy prior to infection. Sasaki's observations on rigorously sterilized diets were referred to earlier. It is confirmed here, however, that genotypic differences do exist, the

$\underline{B}^{14}\underline{B}^{14}$ being much more competent than either the $\underline{B}^{2}\underline{B}^{14}$ or the $\underline{B}^{2}\underline{B}^{2}$. However, the same resolution between the $\underline{B}^{2}\underline{B}^{14}$ and $\underline{B}^{2}\underline{B}^{2}$ as determined by Longenecker *et al.*, was not achieved, possibly due to the fact that the donors were not the F_2 from the $\underline{B}^{2}\underline{B}^{14}$ x $\underline{B}^{2}\underline{B}^{14}$ F_1 cross originally derived from homozygous $\underline{B}^{2}\underline{B}^{2}$ and $\underline{B}^{14}\underline{B}^{14}$ parental strains. Moreover, in CAMP the recipient embryo sex was not determined, and donor sex by and large was not known. These factors may have influenced the significant interaction reported in results, as well.

MES did not distinguish between the genotypes as well as did CAMP. Nor did this assay method distinguish as well between the dietetic and environmental effects. It may measure some things in addition to GVHR. The embryos were harvested at 19 days of incubation at which time the hosts could be considered competent to mount an immune response against the injected cells. Moreover, the fact that replicate analysis could be higher or lower at a given time (but all analyses higher or lower in the same direction) suggests that this analysis is more susceptible to external variables than is CAMP. It suggests, furthermore, that much of the variability resides in the embryonated hosts. It was again confirmed that the sex of the recipient embryo played a part; recipient females giving higher MES than recipient males.

2. Antibody to Human Blood Groups.

There would seem to be a marked tendency toward the $\underline{B}^{2}\underline{B}^{14}$ having a greater ability to acquire antibody of this nature, although not demonstrable as significant using the statistical methods here. It was within the gnotobiotic group in which the more marked response, percentage-wise, in acquisition of antibody to human blood groups occurred amongst the $\underline{B}^{2}\underline{B}^{14}$'s, as did the increase in weight. With increasing numbers of reports indicating the marked influence of diet on the immune response the question comes to mind as to whether or not, in a case such as this, a genetic control over the assimilation of required substances might in fact be the crux of presumed genetic differences in certain aspects of the immune response. Such a question could be answered only by independent study addressed specifically toward it. The impression given by the results of the work here is that the gnotobiotic environment facilitated the adsorption of more essential requirements for both growth and antibody production in the heterozygous $\underline{B}^{2}\underline{B}^{14}$ as compared with the homozygous $\underline{B}^{2}\underline{B}^{2}$ and $\underline{B}^{14}\underline{B}^{14}$.

Again a decreased response as well as titre in antibody detectable with A_1, Rh-negative cells occurred in animals on a tryptophane-deficient diet, both conventional and gnotobiotic. There was a tendency toward a lower titre of anti-A_1 in the conventionals on a conventional diet. A similar transience of antibody, i.e., an increase for a time after two months of age with a subsequent decline, in some instances to zero, as discussed earlier occurred with these animals, as well. It can be seen to a greater or lesser extent with all genotypes irrespective of environment or diet. Genetic control over the acquisition of titre (i.e., amount of) of antibody could not be demonstrated to be statistically significant employing analysis of variance. However, $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ did not acquire antibody to the extent that the $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$ or the $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$ did suggesting an involvement of the $\underline{B}^{\underline{14}}$ allele in a heightened humoral antibody response to continuous stimulation.

3. Weights of Animals

The adverse effect of autoclaving the diet has been discussed earlier. The tryptophane-deficient diet to which animals were transferred after having been on the autoclaved diet obliterated any genotypic differences in weight in the long run. It is difficult to determine why animals on this diet should have been heavier in the conventional environment than in the gnotobiotic unless one invokes the possibility that the fittest survivors (and considerably fewer conventional survived than did gnotobiotic; see Results) derived a certain amount of tryptophane from the organisms which inhabited them. An indigenous population of microorganisms could have been synthesizing tryptophane.

In the conventional environment with "conventional" diet $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$'s weighed less than the other two genotypes. The animals were kept up to the age of approximately seven months only. Longenecker, *et al*., 1972, determined that over a period of eighteen months $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$ survived less well. Whether or not they would ultimately have succumbed in greater frequency here cannot be determined. However, reverting back to the data on animals on the tryptophane-deficient diet the $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$ would appear to have been less sensitive to this type of stress than the $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ (see Results) in both gnotobiotic and conventional environments. This may constitute supportive evidence that survival is not a simple function of the number of $\underline{B}^{\underline{14}}$ (or $\underline{B}^{\underline{2}}$) alleles, albeit the nutritional stress can hardly constitute a desirable experimental condition for measuring longevity. Although the presence of the $\underline{B}^{\underline{2}}$ allele might appear to be beneficial in some circumstances, e.g., in resistance to infections, for instance Marek's Disease (Burmeister and Purchase), the data in this and the previous experiment do not support ascribing any advantage to the presence of the $\underline{B}^{\underline{2}}$ allele in survival. With the exception of the tryptophane-deficient diet no artificial manipulation of the animals took place. They were not deliberately immunized or infected in any way.

E. Effect of Gnotobiosis on Immune Competence of Thymectomized and Non-thymectomized Chickens

1. Graft-versus-Host Reactivity

In testing the function of a given organ or tissue, the classical method of assessment is its extirpation and subsequent observation of any diminished capacity to function in the animal. Inasmuch as GVHR is considered a parameter of thymus dependent lymphoid function, removal of the thymus should presumably bring about decreased GVHR. The first test here using CAMP was in keeping with the expected. In both gnotobiotic and conventional environments embryos differing from the donor at the B histocompatability locus were used so that major histoincompatabilities were involved. The thymectomized animals were less efficient donors. However, in the work of Sheridan, _et al_., referred to earlier, using MES as the method of assay, the reverse held true for minor histoincompatability antigens. Pazderka (personal communication) had confirmed Sheridan's work using outbred embryos in place of those identical at the B histocompatability locus. Hence, the suspicion that the thymectomy induced increase, being reproducible under different experimental conditions, tended to point to some second factor as constituting the cause, possibly a substance or an infectious agent of unknown origin whose presence in the injected whole blood could provoke spleen enlargement in the recipient embryo. Sheridan, Law, and Ruth, 1969, interpreted the fact that, as in the experiments here, the thymectomized donors often give a greater response to minor histoincompability as being the result of variable release from antigen competition by environmental antigen and accordingly minor histoincompatabilities should be more readily detected in gnotobiotic animals (as is the chick embryo) with cells from gnotobiotic donors. In the experiments here a significant difference between thymectomized and non-thymectomized donors from the gnotobiotic environment could not be demonstrated using MES assayed with embryos identical at the B locus. A non-significant tendency existed for higher spleen weights when thymectomized donors were used. The superior competence of the conventional thymectomized donor was confirmed.

An aspect which has not been explored elsewhere in this regard is the possibility that, toxins, and particularly endotoxins from endogenous gram-negative intestinal organisms which must be present in those animals from the conventional environment could play a role. Springer, 1970, has suggested that those components on red cells representing blood groups, which might also be considered as receptor sites, could function in a transport mechanism, for instance, in binding and transporting endotoxins to the liver for detoxification. It is not unreasonable to think that a thymectomized animal might generally, but from an immunological point of view in particular, be deficient in a mechanism of resistance in coping with toxins. Further, if the minor histocompatability sites should represent those receptor sites on the surface of red cells in particular (although on other circulating cells, as well) to which toxins can bind, the enhanced spleen enlargement in recipient embryos identical at the B histocompatability locus might represent an immune response of the host against the toxin. Since the embryos were injected at 11 days of incubation and the spleens from them harvested eight days later on the 19th day of incubation in the case of non-B reactions, sufficient time could have elapsed during which the embryo could have acquired some immunological competence to mount an immune reaction. With a diminished capacity to destroy the toxins immunologically, whole blood from a thymectomized donor could be expected to carry a greater amount of toxin, thereby provoking an enhanced immunological response from the recipient embryo, expressed by spleen enlargement due to proliferation of immunologically competent cells in the spleen. Such an hypothesis is readily amenable to quantitative experimental testing with the germfree animal providing a well controlled "test tube" in which interference from an indigenous population of microorganisms would pose no problem.

The isolator animals used here were not germfree, see Table 3M of the Appendix. At two weeks of age most of the animals became contaminated through the feed with Clostridium perfringens, which "normally" inhabits the intestine of man and animals to a greater or lesser extent at different times. It also produces powerful toxins, largely exotoxins. The presence of this organism may contribute to the tendency of the gnotobiotic thymectomized to be more efficient donors when testing for minor histoincompatabilities, if the above interpretation is correct, and a microbial population which adds both exo- and endotoxin producing organisms in the conventional environment would enhance this effect. This particular reaction might not be apparent in the B histoincompatability reaction due to the intensity of this reaction and the short period of time required to elicit it. This work establishes that exposure to a microbial environment can alter GVHR to non-B antigens but does not give information with which to evaluate the above interpretation and extension of Springer's hypothesis.

2. Acquisition of Antibody to Human Blood Groups

The production of antibody against human blood groups was diminished in this group of experimental animals. It should be noted that the initial diet before transfer to the tryptophane-deficient diet contained soybean (see Materials and Methods for formulae) as a protein source, and may not have been as antigenic, especially after autoclaving, as the chick starter of previous experiments. A few animals whose autopsy reports from the veterinary pathology laboratory were available (conventionally housed animals only) were diagnosed as encephalomalacia due to vitamin E deficiency, a fat soluble vitamin which could have been destroyed in excess with autoclaving the feed.

3. Growth and Weight

Modifications in the diet could have contributed to a poorer assimilation, in the gnotobiotic environment, of the diet used in this experiment. Surgery had no effect on the weight of the animals.

4. Survival

Sheridan, Law and Ruth, 1969, reported no significant effect on survival by thymectomy (or bursectomy) over the unoperated controls. The diet, in this case, could have compounded the surgical effects thus rendering the thymectomized animals the poorest survivors. It would appear that the gnotobiotic environment was a protection for the thymectomized animals. However, there were three times as many thymectomized animals in the gnotobiotic group, a proportion which makes survival difficult to assess in relation to the environmental effect.

F. Effect of Gnotobiosis on Immune Competence of Bursectomized Chickens

1. Chemical Bursectomy

The use of cyclophosphamide as a simple chemical means of bursectomy appeared genuinely unreliable and troublesome. Inconclusive, or lack of, results obtained are discussed with specific tests as they apply. O'Malley, et al., 1969, report protection against death from toxic doses of nitrogen mustards in axenic mice as have White and Claflin, 1963. However, insofar as the nitrogen mustard in the form of cyclophosphamide was administered immediately on hatching and three days following in these experiments it is difficult to rationalize the effect of an intestinal flora on the ultimate target of the cyclophosphamide, the bursal cells, as being involved in those differences which did exist between gnotobiotic and conventionally housed animals. Further, in another species, the mouse, Sasaki has observed an obliteration of the hypersensitive state (considered a parameter of thymus dependent cells) through the use of cyclophosphamide. Hence, the specificity of its action may well be open to question, and the nature of its effects would appear to require much further study.

2. Graft-versus-Host Reactivity

The adolescent, bursectomized animals still presumably under no physiological stress (such as a deficient diet) showed no statistical difference in GVHR competence between germfree and conventionally housed animals in the initial CAMP assay. It is possible that the administration of BCG and subsequent skin testing provoked additional variation. The initial CAMP assay constituted the third time that GVHR was assayed on a given set of experimental animals when diet should not have been an influencing factor; the other two times for the three genotypes, $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$, $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$ and $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$, being three to three and one-half weeks of age, and for the thymectomized and sham operated at four weeks of age. In the thymectomy experiment, gnotobiotic animals were distinctly less efficient donors. In the other two instances differences between gnotobiotic and conventional were non-significant but gnotobiotic donors did not reach the level of efficiency of the conventionally housed.

One would not expect the hypersensitive state to affect GVHR if one accepts the premise that a single cell or cell clone is responsible for the immune response to a single variety of stimulus. But one cannot overlook the fact that an infectious agent could alter by its own growth and metabolism the productivity of host cells. Moreover, the concept of a transfer factor in passaging the hypersensitivity reaction from one individual to another does not necessarily include a predilection for a given lymphocyte identifiable by any experimental means. Hence, one can ask how the hypersensitive state is magnified within an individual; is it proliferation of a given clone of cells, or is a passage of a "transfer factor" from cell to cell involved, as well?

Likewise, the hypersensitive state in tuberculosis, as well as the production of antibodies, has the time-honored enigma or lack of clarity in its responsibility for protection against disease or for pathogenesis of the disease (Horby, et al., 1966, and Minden, et al., 1971).

Hence, it did not seem unreasonable to attempt to evaluate interactions, if any, in cellular mechanisms of immunity while delineating the effects of environmental infectious agents. There are many saprophytic Mycobacteria (as well as fungi) in nature which could elicit the hypersensitive state "naturally".

The first test in doing CAMP pre- and post-skin testing produced very bizarre results which hardly lent themselves to interpretation. The thought that factors such as a recent contamination by a single outside organism in the case of the gnotobiotic animals, as well as a recent gearing up of the immune mechanism to the injected BCG might have been responsible for the variation, influenced the decision to repeat the test. Since the second experiment had the benefit of experience one might consider it more reliable and conclude that the hypersensitive state did not, under the experimental conditions, influence GVHR.

3. Testing for Hypersensitivity

Measurement of hypersensitivity in the avian species and primarily the domestic chicken has been by measuring wattle thickness following injection of antigen (Panigraphi, et al., 1972, Byerly and Dawe, 1972 in delayed hypersensitivity reactions in Marek's disease virus-infected chickens, Jankovic and Isvaneski, 1963, and Warner, Ovary and Kantor, 1971). Another method involving the termination of the animals is inhibition of splenic cell migration with antigen (Morita and Sockawa, 1971). The first method does not permit discrimination of any hemorrhagic reactions, if they occur. The method of Anderson, 1971, of injecting antigen into the wing web, was chosen for this reason. However, in the animals used in these experiments skin thickness, unaccompanied by apparent edema or hemorrhagic spots not attributable to trauma of injection, is the only measure of hypersensitivity. Although it is probably no less crude than that used in most of the literature referred to above it can, nevertheless, be argued that correlation of skin thickness with previous exposure to a sensitizing agent leaves room for doubt as to the exact specificity of the reaction if unaccompanied by other correlative examinations.

With this reservation in mind it can be said that eliciting a measurable hypersensitive response in the conventional animals as opposed to the gnotobiotic is in keeping with the findings of Sasaki, 1974, who was not able to detect by measuring with the migration inhibition test a delayed type hypersensitivity to Salmonella enteriditis in germfree mice until the animals were associated with other bacteria. This can be interpreted as a requirement for some type of adjuvant in the production of measurable delayed type hypersensitivity, provided by the microbial flora of the conventional animals.

4. Capillary and Ouchterlony Precipitation Tests

The shortcomings of most standard serological methods in attempting to detect circulating antibodies to various mycobacterial antigens has been reviewed by Minden *et al.*, 1971. Two factors in particular should be kept in mind regardless of the type of serological test chosen in such cases, namely, that relatively few chemical constituents of the tubercle bacillus are responsible for the induction of circulating antibodies and that satisfactory tests for detecting small amounts of antibody to these have not been developed. The two tests used here were for precautionary purposes with the idea that detectable antibody could well complicate any interpretation of skin test results as reported by Panigraphi *et al.*, 1972.

5. Cunningham Plaque Assay for Antibody Producing Cells

This particular test should be the discriminating analysis as to the effectiveness of both types of bursectomy, the surgical and the chemical. Surgical bursectomy drastically reduced or eliminated completely those splenic cells responsible for a primary response in antibody production against sheep red cells. However, the chemical bursectomy did not produce such definitive effects.

As mentioned previously, it is difficult to arrive at any interpretation involving microbial environment as causative in the inverse effects seen in cyclophosphamide treated gnotobiotic animals (sham operated with respect to surgical bursectomy) as compared with the conventionally housed. That is to say, the number of plaque forming cells increased in gnotobiotic chickens as opposed to a decrease in conventional chickens. One can, however, invoke an age factor in comparing these animals with those of Toivanen, et al., 1972; Linna, Frommel and Good, 1972, who were working with cyclophosphamide treated chickens under two months of age. The chickens used here were about 5½ months of age which should have been well past the time of bursal regression (at 16 weeks of age the bursa is only a rudiment in White Leghorns). If the interpretation of Toivanen, Toivanen, Linna and Good, 1972, is correct in that the postembryonic stem cell responsible for humoral immunity emigrates from the bursa to the bone marrow at the time of bursal regression, and that the spleen and thymus also possess some cells with a functional capacity for the development of humoral immunity, then the cell type being dealt with at age 5½ months in the plaque assay might well be different from the bursal cells affected by cyclophosphamide immediately on hatching. In any event, in the experiments here, the attempt at chemical bursectomy did not parallel the drastic diminution of humoral antibody production by the surgical bursectomy.

As with the GVHR there was a tendency toward fewer plaque forming cells from sham operated animals in the gnotobiotic environment but not to the extent of being significant with the statistical methods employed. This more or less parallels the work of Olson and Wostmann, 1966, in another system where the cellular and humoral immune response of germfree mice stimulated with 7S human gamma globulin and *Salmonella typhimurium* was measured. An adequate, though lesser response to the antigens was found in the germfree as opposed to the conventional mice but plasmacytic cells, blast cells and large lymphocytes showed a proportionately greater increase in the germfree. It was suggested that the fewer competent cells in the germfree animal might be less committed due to the lack of previous antigenic stimulation; hence an efficient source of less differentiated-type cells to process the antibody might be available in the germfree. The work of the same authors on the chicken, 1964, indicated amongst other things that there was a slower decline in circulating antibody after peak production time in the germfree chicken versus the conventional.

An aspect not considered in any of the above work with the germfree chicken, or by Thorbecke, *et al*., 1957, was the correlation between lymphoid organ size and body weight, although organ weights were compared with those of the conventional. Fortuitously, spleen weight and body weight were taken in the experiments described here, and it is considered a corollary to the above discussion that a significant, direct correlation existed between spleen weights and body weight in the gnotobiotic animals but not in the conventional. In short, the conventional environment introduced variables at least in spleen weight through stimulation of presumably the lymphoid system. Gordon, referred to by Thorbecke, et al., 1957, found spleen, thymus and bursa weights of germfree chickens did not differ from the conventional controls. However, in the absence of correlation with body weight in that work it is difficult to compare the two results. It may be worthy of mention that the experience of Coates, 1974 (personal communication) has been similar to those presented here insofar as the body weights of her gnotobiotic and germfree animals have tended to be higher than the conventional.

The diet of the animals doubtless contributed to the relatively undifferentiated histology of the spleens of these young adult animals at the age at which they were terminated, namely, nearly six months of age. However, as observed by Thorbecke, et al., 1957, a sharp distinction between red and white pulp as seen in mammals did not exist. Lymphoid tissue is presumably more diffusely spread throughout the organ than in mammals but predominantly located around the arteries and their branches. It was impossible to say that this held true in these experimental animals but the foci which were observed in the sham operated animals did have a tendency to be located around the arteries. These could be the "secondary nodules" referred to by Thorbecke, sharply demarcated, round and oval accumulations of cells. See Figure 28. Mitotic figures were sometimes present as were macrophages.

It should be kept in mind that the sections were taken from spleens following primary stimulation with sheep red cells. Therefore, it cannot be determined whether these foci would have existed prior to the stimulation or not, particularly in the case of the gnotobiotic sham operated animals which did not, under these circumstances exhibit a detectable difference from the conventional sham operated. Thorbecke, et al., observed fewer "secondary nodules" later in life in germfree as opposed to conventional chickens. One can infer a lymphoidal derivation from the bursa in this instance by virtue of the fact that they did not exist in the bursectomized animals, either gnotobiotic or conventional in keeping with the report by Pierce, Chubb and Long (1966).

6. Titration of Plasmas for Determination of Antibody Against Sheep Red Cells

Although the method of estimation of antibody against both SRBC and human blood groups can at best be termed semi-quantitative, the titrations lean toward greater amounts of circulating antibody in the sham operated gnotobiotic animals. Wostmann and Olson, 1964, reported that in comparison with their conventional counterparts germfree chickens produced 50% less circulating antibody to bovine serum albumin at the maximum response time, as well as having a slower induction time. Neither of these would seem to have been borne out here, albeit the antigenic system is a complex particulate one as opposed to a simple protein. Numerically, more gnotobiotic, sham operated animals developed antibody in a titre of 1/128 or higher than did conventionals. Likewise, the lowest titre amongst the gnotobiotic was 1/64 as opposed to 1/32 with the conventionals. Hence, one would assume that peak production in five days as worked out by Fredericksen, had been achieved or nearly achieved and that the capacity of the gnotobiotic animals to respond with the production of antibody was at least that of the conventional. A better correlation with plaque forming cells would be a quantitative determination of plasma antibody. One conventional sham operated animal produced no detectable antibody whatsoever.

7. Acquisition of Antibody Against Human Blood Groups

As previously the acquisition of antibody against human A_1, Rh-negative red cells was nil in the gnotobiotic animals and minimal against human B, Rh-negative red cells until the injection of SRBC following which a significant response occurred. In the conventional sham operated animals, a picture similar to that of previous occasions occurred, namely, that some antibody against all three blood group entities of the ABH(O) system was acquired by most of the animals, the frequency being anti-A_1 < anti-B < anti-O with a subsequent decrease or disappearance of antibody after the initial increase. Some (but not all) exhibited a second increase by the time of SRBC injection which in turn elicited a relative increase in titre to B cells and O cells in most of the animals and in about one-half the animals to A_1 cells.

That an increase in antibody against human B cells occurred (in both gnotobiotic and conventional sham operated animals) on the injection of SRBC is of particular interest insofar as it implies a determinant or receptor site like or similar to human B blood group on the sheep erythrocyte. Although there has been no blood group system reported in the sheep to be identical with the human B system, red cells are characterized by multiple agglutinogens (Wiener, A.S. and I. Wexler, 1952). That there might be cross-reactivity is, therefore, not surprising. The plasma used here may constitute a reagent for the detection of a certain complex or portion thereof not reported previously on the sheep red cell to which the chicken is free to react by producing abundant antibody as in the case of cross-reacting substances in bacteria, readily detectable with human B, Rh-negative red cells following adsorption with human O, Rh-positive cells. Because the reaction occurs with B cells following adsorption with O cells it cannot be considered as being against species specific determinants shared by all members within the species (in this case human) but rather against the type specific determinant or receptor site known as a blood group encountered only by that percentage of individuals within the species possessing it.

A similarity to the human A complex on sheep red cells has long been known through the detection of heterophile antibodies. If the chicken has a structure or receptor site on its own cells similar to human A, the lesser production of anti-A_1 on injection of SRBC is as would be expected under those circumstances and that which is produced could be considered of heterogeneous origin.

G. General Discussion

This work represents the first comprehensive investigation of the cellular immune response in the avian species under gnotobiotic conditions comparable to investigations in the rodent, mice in particular (Wilson, Sjodin and Bealmear, 1964; Bealmear and Wilson, 1967; Jones, Wilson and Bealmear, 1971). Others, in addition to the above workers, have investigated GVHR in mice and rats. The general agreement that GVHR does occur in the germfree animal but that the severity of the reaction can be affected by external factors such as sensitive recipient age, cell dosage (and in the case of mice irradiation dosage, Walburg and Cosgrove, 1969) is supported by this work with the chicken. Although this work was not specifically designed to measure the effects of nutrition on GVHR and other immune responses, it can be safely concluded said that diet has an effect more profound than microbial status. This is in accord with some work on rodents, but there are reports which differ. Walburg and Cosgrove (1969) found no difference between germfree and conventional donors upon injection of parental spleen cells into F_1 hybrid mice following whole body x-irradiation. Nielsen (1972) did not find a difference in germfree as compared with conventional rats in the reactivity of their cells in mixed lymphocyte culture or GVHR as measured by lymphnode enlargement (in keeping with work by Wilson and Fox, 1971). However, previously Salomon and Lecourt (1966) had found that the source of donor cells played a role, namely, that cells from germfree animals were less efficient. This work leans toward the latter. Although the statistical significance in the difference between gnotobiotic and conventional donors varied, gnotobiotic animals did not attain the same level of donor competence as did their conventionally raised counterparts when tested under a range of diverse experimental conditions and at different ages. In measuring the cellular response in antibody forming cells (to SRBC) the gnotobiotic were again slightly, though not significantly lower. No attempt was made in any of the experiments here to assess any homeostatic factors which could either suppress the expression of immune competence in the intact animals or release an inhibition (Bosma, Makinodan and Walburg, 1966).

It is confirmed, moreover, that cellular immune responses responsible for GVHR are separate from humoral antibody response under conditions approaching the natural, i.e., wherein there was no experimental manipulation or disturbance of the animal. This is evidenced by the fact that a progressive increase with age in GVHR occurred in both gnotobiotic and conventional animals while the natural acquisition of antibody to human blood groups through microbial, dietetic and other physiological stimulation rose and fell. Moreover, this occurred at age approximately four months (and again at about eight months in another species, the English Game Hen, Figure 34). In the chicken this should be the prime of life and the capacity for antibody production should, for all intents and purposes, be at its height.

Why it is not, is therefore, an unknown factor in the assessment of the animal's immune competence.

The gnotobiotic animal provides a unique tool in defining the terms of reference of immunologic responses which are "natural" or which constitute genetic variations of the same. The detection of maternal antibody, presumably of an IgG nature as opposed to IgM, in the young has been referred to, as has the confirmation of the genetic control over GVHR. Another type of immune response, delayed-type hypersensitivity which has some comparable naturally occurring situations has been investigated experimentally on a very limited scale here. The classical system for delayed-type hypersensitivity is the reaction against tuberculin by sensitive individuals. As used here the system brings out the point that variations and/or aberrations of the immune response can occur by the effects of a superimposed indigenous microbial population. Only conventional animals became measurably sensitive to tuberculin.

Contradictions exist in the literature as to the nature of the hypersensitivity reaction. Others have ascribed this phenomenon variably as being a parameter of bursa and of thymus derived development. Warner and Szenberg (1964) claimed bursectomy obliterated the hypersensitive response to tuberculin and vaccinia virus, whereas Cooper, _et al_. (1966) found that thymectomy did so when chickens were sensitized with diphtheria toxoid. In the experiments of Jankovic and Isvaneski (1963) all normal and bursectomized birds responded with the production of experimental allergic encephalomyelitis to the injection of either chicken or bovine spinal cord, as well as to tuberculin inasmuch as *Mycobacterium tuberculosis* was used in the Freund's complete adjuvant. Thymectomized birds exhibited an impaired ability to mount a hypersensitive reaction as measured by the occurrence of allergic encephalomyelitis and skin test reactions to tuberculin. An extension and confirmation of these experiments was performed by Blaw, Cooper and Good (1967). Panigraphi, *et al*. (1972) were unable to confirm an impaired delayed hypersensitivity to tuberculin in White Leghorn chickens subjected to thymectomy within 24 hours of hatching and total body irradiation at three days of age. Indirect evidence indicated an Arthus reaction as being involved.

In a naturally occurring autoimmune disease, spontaneous autoimmune thyroiditis, in the obese strain (OS) of White Leghorn chickens characterized by infiltration of the thyroid gland with large mononuclear cells, and the presence of many germinal centers, as well as circulating antibodies, Wick and Nilsson have provided evidence that bursal cells are heavily involved in the development of the disease. Bursectomy (Wick, *et al*., 1970), *in ovo* and at hatching, decreased the incidence and severity of the disease with accompanying absence of precipitating and hemagglutinating thyroglobulin antibodies associated with the disease. Thymectomy and thymo-bursectomy showed an enhancement effect and a slight suppressive effect respectively (Wick, *et al*., 1970) further supported by the finding that treatment with anti-bursal cell serum suppressed and anti-thymus cell serum enhanced disease development (Wick, Kite and Cole, 1971). Reconstitution of bursectomized-irradiated chickens with autologous bursal cells showed total restoration of thyroiditis (Nilsson and Rose, 1972). Further, Wick (1973) induced experimental allergic encephalomyelitis in hormonally (*in ovo*) bursectomized, OS, chickens without an apparent mutual interaction of the two diseases but did observe, additionally, that encephalomyelitis was not solely thymus-dependent inasmuch as bursectomy (albeit *in ovo*) also had a suppressive effect None of these experiments consider the effect of a microbial population in bringing about the response described.

The gnotobiotic animal could permit the designing of experiments where purposeful additives to a sensitizing agent or organism can be assessed in addition to the relationship of hypersensitivity to thymus or bursal ontogeny and comparison with natural autoimmunity. It could permit the definition of the role of hypersensitivity in some infectious diseases thereby providing an insight into the mechanisms of pathogenicity of the invading organisms. In turn, it could provide a tool for assessing the mechanism of protection which live BCG gives as an immunologic stimulatory agent in increasing tumor resistance (Hanna, Zbar and Rapp, 1972). This work provides data which provoke the need for recognition of the extrinsic influence on immunologic responsiveness by a naturally occurring microbial population.

SUMMARY

1. GVHR in gnotobiotic chickens was lower than in conventionally housed chickens with variation in the significance of the difference interpreted as being related to other extrinsic factors such as nutrition. Gnotobiotic chickens never attained the GVH donor competence of conventionally raised chickens.
2. A genetic difference in donor efficiency between homozygous $\underline{B}^{14}$ and heterozygous and homozygous $\underline{B}^{2}$ was confirmed in that $\underline{B}^{14}\underline{B}^{14}$ showed approximately four times the efficiency of $\underline{B}^{2}\underline{B}^{14}$ and $\underline{B}^{2}\underline{B}^{2}$.
3. A definitive environmental effect on donor efficiency of thymectomized chickens on MES where GVHR to minor histoincompatabilities was measured was established. Conventionally housed thymectomized donors showed increased GVHR. Thymectomy diminished donor competence in GVHR to major $\underline{B}$ histoincompatability.
4. Bursectomy did not influence GVHR of donor cells when no other physiological stress was involved.
5. The hypersensitive state had no effect on GVHR or humoral antibody production in the chicken.
6. Nutrition and diet had a more pronounced effect on immune competence than did microbial status.
7. Heat sterilization of diet had a pronounced detrimental

effect on growth and weight of chickens which could not be corrected by supplementing with vitamins. Correlation between body weight and immune competence could be made in some instances.

8. Thymectomy decreased survival of animals under the experimental conditions as did bursectomy. The gnotobiotic environment tended to favor survival.
9. Initial acquisition by physiological stimulation of "naturally occurring" antibody against the major human ABH(O) blood group system was transient with a second increase in acquisition of antibody occurring after four or five months of age.
10. There was a strong tendency for the heterozygous $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$ chicken to acquire antibody to human blood groups in greater frequency which seemed correlated with body weight.
11. The acquisition of titre of antibody against human A_1, B or H(O) cells tended to be lower in $\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ as compared with $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$ or $\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$.
12. No significant difference in older adult White Leghorns in antibody against A_1, or B, Rh-negative cells could be established although a definite tendency toward greater titres against B cells existed. In young animals acquisition of anti-A_1 was less frequent and in lower titre than anti-B.
13. In older adult White Leghorn chickens no genotypic difference in antibody against human blood groups could

be established.

14. Adult male chickens ganglionectomized at hatching showed a decreased acquisition of antibody toward human blood groups in total as measured in the unadsorbed plasma and the effect was most pronounced in those males which had had the right ganglion or both right and left ganglia removed.
15. The injection of SRBC provoked an increase in antibody against human blood groups in the chicken, as well as to SRBC proper. Of particular interest was the production of anti-B for which a similar blood group is not known in the sheep.
16. Surgical bursectomy drastically reduced and in some instances completely eliminated the ability of both gnotobiotic and conventionally housed animals to produce antibodies to SRBC in a primary response when tested at five and one-half months of age by Cunningham plaque assay and serologically.
17. The use of cyclophosphamide as a means of chemical bursectomy was ineffective.
18. Hypersensitivity to tuberculin was elicited only in conventionally housed animals; an observation interpreted as suggesting the necessity of an adjuvant in bringing about a certain level of hypersensitivity.
19. Recipient female embryo spleens weighed more than recipient male embryo spleens in the MES assay for GVHR.

TABLE 1

HATCHABILITY OF $\underline{B}^{2}\underline{B}^{2}$, $\underline{B}^{2}\underline{B}^{14}$, AND $\underline{B}^{14}\underline{B}^{14}$ EMBRYONATING EGGS CONVENTIONALLY INCUBATED THROUGHOUT AND THOSE TAKEN INTO THE ISOLATOR ON THE 20th DAY OF INCUBATION FOLLOWING STANDARDIZED STERILIZATION PROCEDURE

Hatch # and Date	# Eggs Set (Infertile)*		# Eggs Taken into Isolator	# Hatched in Isolator (%)	# Eggs Held Conventionally	# Eggs Hatched Conventionally
1. May 13 and 14, 1972 **	$\underline{B}^{2}\underline{B}^{2}$	14(7)	7	0 (0)	-	-
	$\underline{B}^{2}\underline{B}^{14}$	24(2)	15	3 (20%)	8	2 (25%)
	$\underline{B}^{14}\underline{B}^{14}$	47(14)	20	0 (0)	13	3 (23%)
	Total	85				
2. May 19 and 20, 1972	$\underline{B}^{2}\underline{B}^{2}$	16(11)	5	0 (0)	-	-
	$\underline{B}^{2}\underline{B}^{14}$	18(4)	10	8 (80%)	4	4 (100%)
	$\underline{B}^{14}\underline{B}^{14}$	20(0)	15	7 (46%)	5	4 (80%)
	Total	56				
3. May 30 and 31, 1972	$\underline{B}^{2}\underline{B}^{2}$	5(4)	-	-	1	1
	$\underline{B}^{2}\underline{B}^{14}$	19(2)	9	4 (44%)	8	7 (87%)
	$\underline{B}^{14}\underline{B}^{14}$	23(3)	10	6 (60%)	10	9 (90%)
	Total	47				
4. June 9, 1972	$\underline{B}^{2}\underline{B}^{2}$	36(6)	15	5 (33%)	15	15 (100%)
5. June 24, 1972	$\underline{B}^{2}\underline{B}^{2}$	12(2)	10	3 (30%)	-	-

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* Number in parenthesis refers to number eggs infertile on candling on the 19th day or early on the 20th day of incubation.

** This group only taken into the isolator on the 19th day of incubation.

TABLE 2

HATCHABILITY OF B^2B^2 EMBRYONATING EGGS TAKEN INTO ISOLATORS FOR THYMECTOMY EXPERIMENT (EXP. 3)

Date Taken into Isolator	Total # Eggs in Isolator	# Eggs Hatched	% Eggs Hatched
1. Jan. 28/73	45*	4	8.8
2. Feb. 5/73	48*	7	14.5
3. Feb. 12/73	44*	5	11
4. Feb. 22/73	36	22	61
5. Mar. 1/73	49	31	63
6. Mar. 12/73	23	13	56

* These groups taken into the isolator on the 19th day of incubation; the other trials on the 20th day of incubation. Trials 4. and 6. represent animals kept and subsequently worked with.

TABLE 3

HATCHABILITY OF B^2B^2 EMBRYONATING EGGS TAKEN INTO ISOLATOR ON THE 20th DAY OF INCUBATION FOR BURSECTOMY EXPERIMENT (EXP. 4)

Date of Hatch and Trial #	Environment	Total # Eggs in Isolator	# Hatched	% Hatched
1. June 25/73	Conventional	--	10/12	83
2. *June 27/73	G.F.**	35	19/35	54
3. July 4/73	G.F.	25	14/25	56
4. July 17/73	a) G.F.	16	11/16	69
	b) Conventional	--	5/6	83

* Isolator for this group became contaminated in several days and animals were conventionalized and used as controls.

** G.F. - germfree.

TABLE 4

MEAN $\log_{10}$ AND ARITHMETIC CONVERSION OF MEAN $\log_{10}$ VALUES OF GVH COMPETENCE OF $\underline{B}^2\underline{B}^2$ GNOTOBIOTIC AND CONVENTIONAL CHICKENS ON A TRYPTOPHANE-DEFICIENT DIET (Gn AND C RESPECTIVELY), AND $\underline{B}^2\underline{B}^2$ CONVENTIONAL CHICKENS ON A CONVENTIONAL DIET (C + C) WITH AGE

(a)	6 wks. - 2 mo.		3 mo.		4.5 mo.	
Gn:						
$\log_{10}$	0.36411	± 0.0361	0.46927	± 0.0489	0.54321	± 0.0412
Arith.	2.3		2.9		3.49	
C:						
$\log_{10}$	0.47618	± 0.0436	0.63255	± 0.0848	0.8699	± 0.0775
Arith.	2.99		4.19		7.41	
C + C:						
$\log_{10}$	-		1.43316	± 0.0412	1.62523	± 0.0547
Arith.	-		27.1		42.19	

(b)	For All First Tests	For All Second Tests	Total Sum
n	82	51	133
mean			
$\log_{10}$	1.23575	1.29837	1.25976
Arith.	16.82	19.89	18.99

(a) CAM pock assay with standard error with arithmetic conversion of mean $\log_{10}$.

(b) Replicate analyses of CAM pock assays which include both Gn and C + C chickens.

TABLE 4 (CONTINUED

(c)	2.5 mo.		4 mo.	
Gn: Female				
$\log_{10}$	1.44137	± 0.146	1.66211	± 0.0608
Arith.	27.64		45.93	
Male				
$\log_{10}$	1.34897	± 0.089	1.40489	± 0.052
Arith.	22.33		25.40	
C: Female				
$\log_{10}$	1.6561	± 0.0547	1.72176	± 0.1077
Arith.	45.30		52.58	
Male				
$\log_{10}$	1.53284	± 0.0854	1.70582	± 0.1063
Arith.	34.89		50.80	
C + C: Female				
$\log_{10}$	1.70833	± 0.0412	1.70726	± 0.0678
Arith.	51.09		50.97	
Male				
$\log_{10}$	1.53839	± 0.04	1.57628	± 0.0566
Arith.	34.58		37.70	
Uninoc:				
Female $\log_{10}$	1.01695 (10.4)	n = 67		
Male $\log_{10}$	1.04955 (11.21)	n = 64		

(c) MES giving male and female recipient embryo mg. embryonic spleen weight, as well as the uninoculated controls with arithmetic conversion of mean $\log_{10}$ values.

TABLE 5

SURVIVAL IN DAYS OF $\underline{B^2}\underline{B^2}$ GNOTOBIOTIC AND CONVENTIONAL LEGHORNS ON TRYPTOPHANE-DEFICIENT DIET, AND CONVENTIONAL ON CONVENTIONAL DIET

Chick #	Environment/Diet	Life-Span
24018	Gn/TD	149 d.+
24017	Gn/TD	149 d.+
24016	Gn/TD	149 d.+
24015	Gn/TD	6 d.
24014	Gn/TD	149 d.+
24013	Gn/TD	149 d.+
24012	Gn/TD	149 d.+
24038	Conventional/TD	42 d.
24039	Conventional/TD	75 d.
24040	Conventional/TD	121 d.
24041	Conventional/TD	57 d.
24042	Conventional/TD	149 d.+
24043	Conventional/TD	149 d.+
24044	Conventional/TD	70 d.
24019	Gn/TD	139 d.+
24020	Gn/TD	139 d.+
24021	Gn/TD	139 d.+
24027	Gn/TD	139 d.+
24028	Gn/TD	139 d.+
24300	Conventional/Con.	139 d.+
24299	Conventional/Con.	139 d.+
24298	Conventional/Con.	139 d.+
24297	Conventional/Con.	139 d.+
24350	Conventional/Con.	139 d.+

+ indicates age in days at time at which the experiment was terminated.

TD = tryptophane-deficient diet.

Con. = conventional diet.

TABLE 6

CONTROL HUMAN O, Rh-POSITIVE PLASMA TITRATIONS WITH HUMAN A_1, Rh-NEGATIVE, AND HUMAN B, Rh-NEGATIVE RED BLOOD CELLS

	Reaction with A_1, Rh-Neg. Cells	B, Rh-Neg. Cells
For Plasma #1:		
	n = 14	n = 14
	Mean = 3.07 (±0.021)	Mean = 3.10 (±0.015)
	Range = 2.0 to 4.0 (2.0)	Range = 2.5 to 3.5 (1.0)
	C.V. = 16%	C.V. = 11.9%
For Plasma #2:		
	n = 28	n = 28
	Mean = 6.21 (±0.007)	Mean = 3.46 (±0.021)
	Range = 5.0 to 7.5 (2.5)	Range = 1.5 to 7.5 (6.0)
	C.V. = 5.3%	C.V. = 21.8%

TABLE 7

AGGLUTINATION TITRATION FOR ANTIBODY AGAINST A_1, AND B, Rh-NEGATIVE HUMAN RED BLOOD CELLS AND O, Rh-POSITIVE RED CELLS IN VARIOUS GENOTYPES OF ADULT WHITE LEGHORN CHICKENS

		Adsorbed Plasma (3X)			Unadsorbed Plasma		
Genotype		Anti-A_1	Anti-B	Anti-(O)	Anti-A_1	Anti-B	Anti-(O)
$\underline{B}^{2}\underline{B}^{2}$	1.	6.0	4.0	0.5	7.5	3.0	2.0
	2.	1.5	0.0	0.0	NSQ**		
	3.	3.5	3.0	0.0	5.0	5.0	2.5
$\underline{B}^{2}\underline{B}^{14}$	1.	1.5	0.0	0.0	NSQ**		
	2.	3.5	6.0	0.0	8.0+	8.0+	8.0+
	3.	3.0	1.5	0.0	NSQ**		
$\underline{B}^{14}\underline{B}^{14}$	1.	1.5	0.0	0.0	NSQ**		
$\underline{B}^{13}\underline{B}^{13}$	1.	4.0	0.0	0.0	6.0	3.0	3.0
	2.	4.0	1.0	0.0	4.0	1.0	1.0
$\underline{B}^{2}\underline{B}^{15}$	1.	1.5	4.5	0.0	NSQ**		

** Not sufficient quantity for unadsorbed titrations.

TABLE 7 (CONTINUED)

Genotype	Adsorbed Plasma Anti-A_1	Adsorbed Plasma Anti-B	Adsorbed Plasma Anti-(O)	Unadsorbed Plasma Anti-A_1	Unadsorbed Plasma Anti-B	Unadsorbed Plasma Anti-(O)
$\underline{B}^{\underline{21}}$ homo- and heterozygotes (4X absorbed):						
1.	2.5	4.5	0.0	6.5	7.0	7.0
2.	1.0	1.5	0.0	5.0	5.0	5.0
3.	1.5	2.0	0.0	5.5	6.0	5.5
4.	1.0	1.0	0.0	6.5	6.5	6.0
5.	3.0	4.5	0.0	8.0+	8.0+	8.0+
6.	3.0	4.0	0.0	7.5	7.5	7.5
7.	1.0	3.5	0.0	5.0	6.0	6.0
8.	2.0	4.0	0.0	8.0+	8.0+	8.0+
9.	0.0	2.5	0.0	6.0	7.0	6.0
10.	2.0	3.0	0.0	6.0	7.5	6.5
11.	0.5	1.5	0.0	4.5	4.5	4.5
12.	3.5	4.0	0.0	8.0+	8.0+	7.5
13.	4.0	4.0	0.0	8.0+	8.0+	7.5
14.	2.0	3.0	0.0	6.5	7.0	6.0
15.	0.0	3.0	0.0	5.5	5.5	4.0
16.	0.0	1.5	0.0	3.5	6.5	4.5
17.	1.5	3.0	0.0	4.5	5.0	5.0
18.	0.0	0.0	0.0	3.5	4.5	6.0
19.	2.0	1.0	0.0	4.0	4.0	4.5
n	29	29	29	24	24	24
Mean	2.08 (±0.050)	4.01 (±0.048)	0.00 (±0.00)	5.93 (±0.024)	5.89 (±0.041)	6.41 (±0.044)
Range	0.0 to 6.0	0.0 to 6.0	0.0	3.5 to 8.0	1.0 to 8.0	1.0 to 8.0

TABLE 8

MEAN $\log_{10}$ VALUES OF CAMP GIVEN FOR THE THREE GENOTYPES, $\underline{B}^2\underline{B}^2$, $\underline{B}^2\underline{B}^{14}$ AND $\underline{B}^{14}\underline{B}^{14}$ AT AGES 3 TO 7 DAYS, 3 TO 4 WEEKS AND 3 MONTHS. VALUES OF n REFER TO THE NUMBER OF RECIPIENT EMBRYOS USED

Age	Genotype	Gn	Conv.	Mean Gn + Conv.
3-7 d.	$\underline{B}^2\underline{B}^2$	0.0565 (n=75)	0.0815 (n=81)	0.0694
	$\underline{B}^2\underline{B}^{14}$	0.0562 (n=62)	0.0822 (n=94)	0.0719
	$\underline{B}^{14}\underline{B}^{14}$	0.0288 (n=27)	0.0995 (n=105)	0.0851
3-4 wks.	$\underline{B}^2\underline{B}^2$	0.9489 (n=49)	0.9416 (n=136)	0.9435
	$\underline{B}^2\underline{B}^{14}$	0.8955 (n=178)	1.1433 (n=28)	0.9292
	$\underline{B}^{14}\underline{B}^{14}$	1.2523 (n=150)	1.3179 (n=35)	1.2646
3 mon.	$\underline{B}^2\underline{B}^2$	0.5421 (n=72)	0.5630 (n=16)	0.5459
	$\underline{B}^2\underline{B}^{14}$	0.8383 (n=73)	0.9639 (n=48)	0.8881
	$\underline{B}^{14}\underline{B}^{14}$	0.8388 (n=85)	0.9948 (n=77)	0.9129

TABLE 9

MEAN $\log_{10}$ CAMP AND MES VALUES OF REPLICATE TESTS ON GNOTOBIOTIC AND CONVENTIONAL $\underline{B}^2\underline{B}^2$, $\underline{B}^2\underline{B}^{14}$ AND $\underline{B}^{14}\underline{B}^{14}$ CHICKENS

CAMP:

Environment	Genotype	Animal #	1st Test	2nd Test	Total Mean
Gn	$\underline{B}^2\underline{B}^2$	26851	0.5764	0.6621	0.6248 (n=23)
		26854	0.7928	0.9894	0.8954 (n=23)
	$\underline{B}^2\underline{B}^{14}$	26545	0.8560	0.4310	0.6242 (n=22)
		26546	0.9299	0.8310	0.8659 (n=17)
	$\underline{B}^{14}\underline{B}^{14}$	26530	1.1000	0.8283	0.9271 (n=20)
Conv..	$\underline{B}^2\underline{B}^2$	24023/26	0.7183	0.5231	0.6089 (n=25)
		26824/25	1.0602	0.7100	0.8927 (n=23)
	$\underline{B}^2\underline{B}^{14}$	24029/30	0.8592	0.9224	0.8953 (n=21)
	$\underline{B}^{14}\underline{B}^{14}$	24033	1.2605	1.0852	1.1569 (n=22)
		26569/70	1.3345	0.7926	1.0961 (n=25)

Environmental effect: F-distribution for $F_{1,215} = 5.5438$, $0.010 < p < 0.025$.

Genotypic effect: F-distribution for $F_{2,215} = 10.3102$, $p << 0.001$.

TABLE 9 (CONTINUED)

MES:

Environment	Genotype	Animal #	1st Test	2nd Test	Total Mean
Gn	$\underline{B}^{2}\underline{B}^{2}$	26854	1.6824	1.4555	1.5586 (n=22)
	$\underline{B}^{2}\underline{B}^{14}$	26535	1.6764	1.3998	1.5700 (n=13)
	$\underline{B}^{14}\underline{B}^{14}$	26537	1.7786	1.6897	1.7267 (n=12)
		26541	1.9792	1.7413	1.8899 (n=16)
C	$\underline{B}^{2}\underline{B}^{2}$	26824	1.5611	1.2384	1.4400 (n=16)

TABLE 10

MEAN $\log_{10}$ CAMP OF GNOTOBIOTIC (Gn) AND CONVENTIONALLY HOUSED (C) $\underline{B}^{2}\underline{B}^{2}$, $\underline{B}^{2}\underline{B}^{14}$ AND $\underline{B}^{14}\underline{B}^{14}$ ON A TRYPTOPHANE-DEFICIENT DIET, AND CONVENTIONALLY HOUSED ON A CONVENTIONAL DIET (C+C) AT 6 MONTHS OF AGE

(a)	Genotype	Gn	C	C+C	Mean Gn, C and C+C
	$\underline{B}^{2}\underline{B}^{2}$	0.6269 (±0.015) $4.2\overline{4}$	0.7679 (±0.022) $5.8\overline{5}$	1.1826 (±0.026) $15.\overline{2}3$	0.8249 6.68
	$\underline{B}^{2}\underline{B}^{14}$	0.5993 (±0.016) $3.9\overline{8}$	0.6960 (±0.023) $4.9\overline{7}$	1.1021 (±0.036) $12.\overline{6}6$	0.7487 5.59
	$\underline{B}^{14}\underline{B}^{14}$	0.9241 (±0.02) $8.4\overline{0}$	1.1725 (±0.016) $14.8\overline{8}$	1.9505 (±0.018) $89.\overline{2}1$	1.2599 18.20

(b)	Genotype	Tryp-def. Diet	Conven. Diet	Total Mean
	$\underline{B}^{2}\underline{B}^{2}$	0.6790 (n=130) 4.78	1.1826 (n=53) 15.23	0.8249 (n=183) 6.68
	$\underline{B}^{2}\underline{B}^{14}$	0.6399 (n=143) 4.36	1.1021 (n=44) 12.66	0.7487 (n=187) 5.59
	$\underline{B}^{14}\underline{B}^{14}$	1.0090 (n=160) 10.21	1.9505 (n=58) 89.21	1.2599 (n=218) 18.20

(a) Genotypes compared with environment and treatment with arithmetic conversion of mean $\log_{10}$.

(b) Genotypes grouped according to diet with arithmetic conversions of mean $\log_{10}$.

TABLE 10 (CONTINUED)

(c)	Genotype	Gnotobiotic	Conventional
	$\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$	0.6269 4.24	0.9855 9.67
	$\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$	0.5993 3.98	0.9678 9.29
	$\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$	0.9241 8.40	1.5718 37.32

(d)	Environment/Diet	$\underline{B}^{\underline{2}}\underline{B}^{\underline{2}}$ + $\underline{B}^{\underline{2}}\underline{B}^{\underline{14}}$	$\underline{B}^{\underline{14}}\underline{B}^{\underline{14}}$	Total Mean
	Gn	0.6130 (n=165) 4.1	0.9241 (n=105) 8.4	0.7340 (n=270) 5.42
	C	0.7280 (n=108) 5.35	1.1725 (n=55) 14.88	0.8780 (n=163) 7.55
	C+C	1.1461 (n=97) 14.00	1.9505 (n=58) 89.21	1.4471 (n=155) 28.00

(c) Genotypes grouped according to gnotobiotic and conventional environment only with arithmetic conversions of mean $\log_{10}$.

(d) Environment and treatment data grouped according to the presence of the $\underline{B}^{\underline{2}}$ allele versus its absence with arithmetic conversions of mean $\log_{10}$.

10 (CONTINUED)

(e)	Environment/Diet	$B^{14}B^{14} + B^{2}B^{14}$	$B^{2}B^{2}$
	Gn	0.7807 (n=188) 6.04	0.6289 (n=82) 4.24
	C	0.9239 (n=115) 8.39	0.7669 (n=48) 5.85
	C+C	1.5845 (n=102) 38.32	1.1826 (n=53) 15.23

(e) Environment and treatment data grouped according to the presence of the B^{14} allele versus its absence with arithmetic conversions of mean $\log_{10}$.

TABLE 11

OVERALL RESPONSE OF $\underline{B}^{2}\underline{B}^{2}$, $\underline{B}^{2}\underline{B}^{14}$ AND $\underline{B}^{14}\underline{B}^{14}$ GENOTYPIC LEGHORNS IN ANTIBODY PRODUCTION TO HUMAN A_1, AND B, Rh-NEGATIVE AND H(O), Rh-POSITIVE SURFACE RECEPTORS EXPRESSED AS RESPONDERS VERSUS NON-RESPONDERS, R/NR

Environment:		Gn			C			C+C		
Blood Group:		0	B	A_1	0	B	A_1	0	B	A_1
$\underline{B}^{2}\underline{B}^{2}$	R:	2	1	0	2	2	0	5	5	5
	NR:	5	6	7	9	9	11	0	0	0
	R/NR:	2/5	1/6	0/7	2/9	2/9	0/11	5/0	5/0	5/0
	%R:	28.5	14.3	0.0	18.2	18.2	0.0	100	100	100
$\underline{B}^{2}\underline{B}^{14}$	R:	6	2	1	5	5	1	5	5	5
	NR:	9	13	14	3	3	7	0	0	0
	R/NR:	6/9	2/13	1/14	5/3	5/3	1/7	5/0	5/0	5/0
	%R:	40	13.3	6.7	62.5	62.5	14.3	100	100	100
$\underline{B}^{14}\underline{B}^{14}$	R:	2	1	0	7	7	2	5	5	5
	NR:	10	11	12	4	4	9	0	0	0
	R/NR:	2/10	1/11	0/12	7/4	7/4	2/9	5/0	5/0	5/0
	%R:	16.7	8.3	0.0	63.6	63.6	18.2	100	100	100

TABLE 12

ACQUISITION OF ANTI-H(O) AND ANTIBODY TO ANY SPECIES DETERMINANTS WITH AGE IN GNOTOBIOTIC WHITE LEGHORNS

Age:		2 mo.	3 mo.	4 mo.	5 mo.	6 mo.	7 mo.
B^2B^2	26851	0.0	0.0	4.0	0.0	0.0	0.0
	26854	3.0	0.0	5.0	7.0	7.5	7.5
B^2B^{14}	24010/11	0.0	0.0	1.0	0.0	0.0	0.0
	26544	0.0	3.0	5.0	4.0	3.0	N.D.
	26545	0.0	0.0	2.0	0.0	2.0	N.D.
	26546	0.0	0.0	0.0	5.0	6.0	N.D.
	26533	0.0	1.0	0.0	0.0	0.0	N.D.
$B^{14}B^{14}$	26528	0.0	5.0	5.0	6.0	7.0	N.D.
	26541	4.0	1.0	0.0	0.0	0.0	N.D.

TABLE 13a

ACQUISITION OF ANTI-A_1, Rh-NEGATIVE BY CONVENTIONAL ANIMALS ON A TRYPTOPHANE-DEFICIENT DIET

Age:		2 mo.	3 mo.	4 mo.	5 mo.	6 mo.	7 mo.
$B^{2}B^{14}$	26565/66	0.0	0.0	1.0	0.0	N.D.	N.D.
$B^{14}B^{14}$	24031	0.0	1.0	1.0	0.5	0.0	N.D.

TABLE 13b

ACQUISITION OF ANTI-B, BY CONVENTIONAL ANIMALS ON A TRYPTOPHANE-DEFICIENT DIET

Age:		2 mo.	3 mo.	4 mo.	5 mo.	6 mo.	7 mo.
B^2B^2	24023/24	0.0	0.0	1.0	0.0	0.0	N.D.
	26824/25	2.0	1.0	0.0	0.0	0.0	0.0
B^2B^{14}	26567/68	0.0	4.0	N.D.	(dead)	N.D.	N.D.
	26573/74	0.0	3.0	0.0	0.0	0.0	0.0
	26575/76	2.0	0.0	0.0	1.0	0.0	0.0
	26565/66	0.0	2.0	4.0	N.D.	1.0	0.0
	24029/30	0.0	0.0	0.0	N.D.	3.0	4.0
$B^{14}B^{14}$	26569/70	0.0	4.0	1.0	0.0	0.0	0.0
	26516/17	0.0	0.0	3.0	N.D.	(dead)	N.D.
	24031	0.0	0.0	1.0	3.0	0.0	N.D.
	24033	0.0	2.0	2.0	3.0	0.0	N.D.
	26563/64	0.0	2.0	1.0	N.D.	(dead)	N.D.
	26579/80	0.0	2.0	5.0	N.D.	(dead)	N.D.
	26581/82	1.0	3.0	4.0	N.D.	(dead)	N.D.

TABLE 13c

ACQUISITION OF ANTI-H(O) AND ANTIBODY TO ANY SPECIES DETERMINANTS WITH AGE IN CONVENTIONAL ANIMALS ON A TRYPTOPHANE-DEFICIENT DIET

Age:		2 mo.	3 mo.	4 mo.	5 mo.	6 mo.	7 mo.
$\underline{B}^2\underline{B}^2$	24023/26	0.5	0.0	2.0	3.0	0.0	N.D.
	26824/25	2.0	2.0	0.0	0.0	0.0	N.D.
$\underline{B}^2\underline{B}^{14}$	26567/68	0.0	4.5	N.D.	(dead)	N.D.	N.D.
	26573/74	0.0	4.0	2.0	0.0	0.0	N.D.
	26575/76	2.0	0.0	2.0	4.0	0.0	0.0
	26565/66	0.0	2.5	6.0	N.D.	3.0	0.0
	24029/30	0.0	0.0	1.0	N.D.	4.5	6.0
$\underline{B}^{14}\underline{B}^{14}$	26569/70	0.0	5.5	3.0	0.0	0.0	0.0
	26516/17	0.0	0.0	4.0	N.D.	(dead)	N.D.
	24031	0.0	3.0	4.0	4.0	1.0	N.D.
	24033	0.0	3.0	3.0	5.5	0.0	N.D.
	26563/64	0.0	3.5	2.0	N.D.	(dead)	N.D.
	26579/80	0.0	3.5	6.0	N.D.	(dead)	N.D.
	26581/82	0.5	4.0	5.0	N.D.	(dead)	N.D.

TABLE 14

ACQUISITION OF ANTI-A$_1$ (Rh-NEGATIVE) BY CONVENTIONAL $\underline{B}^2\underline{B}^2$, $\underline{B}^2\underline{B}^{14}$ AND $\underline{B}^{14}\underline{B}^{14}$ CHICKENS ON A CONVENTIONAL DIET

Age:			2 mo.	3 mo.	4 mo.	5 mo.	6 mo.	7 mo.
$\underline{B}^2\underline{B}^2$	26826/27		4.0	5.0	N.D.	3.0	3.0	0.0
	26828/29		0.0	0.0	N.D.	1.0	1.0	0.0
	26930/31		0.0	0.0	N.D.	2.0	2.0	0.0
	26832/33		0.0	3.0	N.D.	3.0	3.0	0.0
	26834/35		0.0	3.0	N.D.	1.0	0.0	1.0
		$\overline{X}$	0.8	2.2		2.0	1.8	0.2
$\underline{B}^2\underline{B}^{14}$	26593		3.0	4.0	4.0	4.0	N.D.	(dead)
	26594		N.D.	2.0	6.0	N.D.	(dead)	N.D.
	26595		1.0	1.0	4.0	1.0	1.0	0.0
	26597		N.D.	1.0	3.0	3.0	3.0	1.0
	26599		N.D.	1.0	1.0	3.0	2.0	1.0
		$\overline{X}$	2.0	1.8	3.6	2.75	2.0	0.67
$\underline{B}^{14}\underline{B}^{14}$	24034		N.D.	3.0	4.0	4.0	4.0	2.0
	24035		N.D.	1.0	3.0	6.0	5.0	3.0
	26585		N.D.	0.0	3.0	5.0	6.0	3.0
	26587		N.D.	0.0	0.0	5.0	4.0	4.0
	26589		N.D.	0.0	2.0	2.0	3.0	1.0
		$\overline{X}$		0.8	2.4	3.2	4.4	2.0

TABLE 15

ACQUISITION OF ANTI-B (Rh-NEGATIVE) BY CONVENTIONAL B^2B^2, B^2B^{14} AND $B^{14}B^{14}$ CHICKENS ON A CONVENTIONAL DIET

Age:		2 mo.	3 mo.	4 mo.	5 mo.	6 mo.	7 mo.
B^2B^2	26826	0.0	4.0	N.D.	2.0	3.0	0.0
	26828	1.0	4.0	N.D.	5.0	4.0	4.0
	26830	3.0	2.0	N.D.	0.0	1.0	1.0
	26832	2.0	3.0	N.D.	2.0	3.0	1.0
	26834	1.0	4.0	N.D.	5.0	0.0	1.0
	$\bar{X}$	1.4	3.4		2.8	2.2	1.4
B^2B^{14}	26593	5.0	6.0	6.0	5.0	N.D.	(dead)
	26594	2.0	2.0	6.0	N.D.	(dead)	N.D.
	26595	4.0	3.0	4.0	2.0	1.0	0.0
	26597	2.0	2.0	2.0	1.0	1.0	0.0
	26599	0.0	1.0	5.0	3.0	1.0	1.0
	$\bar{X}$	1.8	2.8	4.6	2.75	1.0	0.3
$B^{14}B^{14}$	24034	0.0	0.0	4.0	5.0	5.0	2.0
	24035	1.0	2.0	4.0	4.0	3.0	1.0
	26585	0.0	5.0	4.0	5.0	1.0	1.0
	26587	N.D.	0.0	2.0	3.0	2.0	1.0
	26589	2.0	2.0	5.0	1.0	1.0	1.0
	$\bar{X}$	0.5	2.4	3.8	3.6	2.4	1.2

TABLE 16

ACQUISITION OF ANTI-H(O) AND ANTIBODY TO ANY SPECIES DETERMINANTS IN CONVENTIONAL $\underline{B}^2\underline{B}^2$, $\underline{B}^2\underline{B}^{14}$ AND $\underline{B}^{14}\underline{B}^{14}$ CHICKENS ON A CONVENTIONAL DIET

Age:		2 mo.	3 mo.	4 mo.	5 mo.	6 mo.	7 mo.
$\underline{B}^2\underline{B}^2$	26826	5.0	5.0	N.D.	3.0	4.0	1.0
	26828	2.0	6.0	N.D.	7.0	5.0	5.0
	26530	5.0	3.0	N.D.	4.0	4.0	1.0
	26532	1.0	6.0	N.D.	5.0	5.0	3.0
	26534	3.0	4.0	N.D.	7.0	4.0	2.0
	$\bar{X}$	3.2	4.8		5.2	4.4	2.4
$\underline{B}^2\underline{B}^{14}$	26593	7.0	7.0	7.0	7.0	N.D.	(dead)
	26594	3.0	3.0	6.0	N.D.	(dead)	N.D.
	26595	6.0	5.0	7.0	3.0	3.0	0.0
	26597	3.0	N.D.	4.0	3.0	8.0	3.0
	26599	2.0	3.0	6.0	4.0	3.0	1.0
	$\bar{X}$	4.2	4.5	6.0	4.25	4.7	1.3
$\underline{B}^{14}\underline{B}^{14}$	24034	4.0	3.0	5.0	N.D.	6.0	5.0
	24035	3.0	4.0	5.0	7.0	6.0	3.0
	26585	5.0	5.0	6.0	5.0	5.0	3.0
	26587	2.0	5.0	6.0	5.0	5.0	5.0
	26589	3.0	4.0	7.0	4.0	3.0	2.0
	$\bar{X}$	3.4	4.2	5.8	5.25	5.0	3.6

TABLE 17a

MEAN WEIGHTS IN GRAMS OF $\underline{B}^{2}\underline{B}^{2}$, $\underline{B}^{2}\underline{B}^{14}$ AND $\underline{B}^{14}\underline{B}^{14}$ GENOTYPES PRE- AND POST-TRANSFER TO A TRYPTOPHANE-DEFICIENT (T.D.) DIET IN THE GNOTOBIOTIC AND CONVENTIONAL ENVIRONMENT, AS WELL AS PRIOR TO TERMINATION.

		5-7 d.			
		Pre-T.D.	Post-T.D.	95-105 d.	Final
Gn:	$\underline{B}^{2}\underline{B}^{2}$	102	93	111	136
	$\underline{B}^{2}\underline{B}^{14}$	141	146	138	174
	$\underline{B}^{14}\underline{B}^{14}$	95	97	111	143
C:	$\underline{B}^{2}\underline{B}^{2}$	153	122	138	170
	$\underline{B}^{2}\underline{B}^{14}$	112	104	132	167
	$\underline{B}^{14}\underline{B}^{14}$	104	91	123	180
Total $\overline{X}$		118	109	126	162
Mean $\log_{10}$		2.0684	2.0385	2.09823	2.2095

TABLE 17b

MEAN WEIGHTS OF CONVENTIONAL ANIMALS OF GENOTYPES AS IN TABLE 17a WHICH WERE ON A CONVENTIONAL DIET THROUGHOUT

	5-7 d.	26 d.	102-109 d.	200+ d.
B^2B^2	46	235	1165	1625
B^2B^{14}	58	237	1130	1560
$B^{14}B^{14}$	44	188	1122	1440

TABLE 18

MEAN $\log_{10}$ WITH THE ARITHMETIC CONVERSION OF THE MEAN FOR CAM POCKS FROM THYMECTOMIZED AND NON-THYMECTOMIZED GNOTOBIOTIC AND CONVENTIONAL CHICKENS (GIVEN AS THE NUMBER OF POCKS PER 0.1 ml. OF DONOR WHOLE BLOOD)

(a) CAM pocks from all available donors:

	T̸	T
Gn:	*0.8160 ±0.035 (n=207)	1.0955 ±0.035 (n=123)
**	6.55	12.46
C:	0.9185 ±0.066 (n=56)	1.2900 ±0.069 (n=69)
	8.28	19.5

(b) Replicate tests:

			1st Test	2nd Test	Mean
Gn:	T̸	29203	*0.9422 (n=15)	1.0537 (n=9)	0.9840
			** 8.77	11.32	9.64
		29206	0.9141 (n=19)	0.5297 (n=9)	0.7905
			8.19	3.39	6.17
	T	26873	1.1451 (n=14)	1.0380 ~~(n=10)~~	1.1005
			14.00	10.91	12.6
		29209	1.1342 (n=15)	1.2070 (n=8)	1.1595
			13.62	16.41	14.44
		29205	1.1984 (n=16)	1.4240 (n=5)	1.2521
			15.79	26.55	17.86

n refers to the number of embryos involved.
* mean $\log_{10}$.
** arithmetic conversion of the mean.
T̸ = thymectomized.
T = non-thymectomized (sham-operated).

TABLE 19

NUMBERS OF LYMPHOCYTES PER CUBIC MILLIMETER OF WHOLE BLOOD FROM THYMECTOMIZED AND NON-THYMECTOMIZED DONORS OF GNOTOBIOTIC AND CONVENTIONALLY HOUSED CHICKENS.PRIOR TO AND FOLLOWING THE TIME OF CONVENTIONALIZATION OF THE GNOTOBIOTIC

			# Lymphocytes/mm^3	
Chicken # and Treatment			Pre-conv.	Post-conv.
Gn:	29206	T̸	8,000	N.D.
	29216	"	5,000	10,000
	29201	"	16,000	N.D.
	29203	"	23,000	13,000
	29202	"	4,000	N.D.
	29217	"	9,000	5,000
	26872	"	14,000	11,000
	29290	"	17,000	N.D.
	29284	"	7,000	N.D.
	29248	"	9,000	N.D.
	29294	"	9,000	3,000
	29280	"	9,000	N.D.
	26873	T	8,000	9,000
	29209	"	14,000	6,000
	29205	"	14,000	N.D.
	26875	"	7,000	14,000
	29296	"	8,000	N.D.
	29276	"	13,000	4,000
	29288	"	14,000	N.D.
	29282	"	11,000	13,000
C:	29340	T̸	2,000	7,000
	29330	"	14,000	12,000
	29390	T	17,000	11,000
	29338	"	9,000	10,000
	29326	"	12,000	17,000
	29332	"	14,000	11,000
	29392	"	7,000	13,000

TABLE 20

ACQUISITION OF ANTIBODY AGAINST HUMAN O, Rh-POSITIVE CELLS

(a) Gnotobiotic thymectomized (T̸) and sham-operated (T)

		2.5 mo.	3 mo.	3.5 mo.	4 mo.	4.5 mo.
T̸	1.	0.0	1.0	0.0	0.0	0.0
	2.	3.0	2.0	0.0	0.0	0.0
T	1.	0.0	0.0	1.0	0.0	0.0
	2.	0.0	1.0	0.0	0.0	0.0
	3.	0.0	0.0	2.0	0.0	0.0

(b) Conventional thymectomized and sham-operated

		1.5 mo.	2 mo.	2.5 mo.	3 mo.	4 mo.
T̸	1.	2.0	1.0	0.0	3.0	0.0
T	1.	0.0	2.0	0.0	0.0	0.0
	2.	0.0	1.0	0.0	0.0	4.0
	3.	1.0	0.0	0.0	0.0	0.0
	4.	2.0	0.0	0.0	0.0	0.0

TABLE 21

WEIGHTS IN GRAMS OF $\underline{B}^2\underline{B}^2$ THYMECTOMIZED (T̸) AND SHAM OPERATED (T) GNOTOBIOTIC (Gn) AND CONVENTIONAL (C) LEGHORNS

First Hatch:

Gn:

Treatment	Chick #		32 d.	42 d.	84 d.
T̸	29213		201	---	dead at 56 d.
	29211		227	---	dead at 70 d.
	26872		128	133	133
	29201		156	150	150
	29202		227	232	224
	29203		172	145	156
	29206		204	167	159
	29216		198	196	215
	29217		187	204	196
		$\bar{X}$	189	175	176
T	26873		170	150	150
	26875		163	170	155
	29205		201	164	164
	29209		193	187	184
		$\bar{X}$	182	168	163

TABLE 21 (CONTINUED)

Second Hatch:

Gn:

Treatment	Chick #	62 d.	78 d.	92 d.
$\bar{T}$	29248	120	dead at 74 d.	
	29280	150	130	130
	29284	177	---	162
	29290	244	132	181
	29294	159	142	133
	$\bar{X}$	170	135	152
T	29276	230	---	232
	29282	198	198	192
	29288	179	153	dead at 83 d.
	29296	170	136	dead at 94 d.
	$\bar{X}$	194	162	212

C:

Treatment	Chick #	53 d.	78 d.
$\bar{T}$	29330	188	205
	29340	210	212
	29386	300	dead at 64 d.
	29388	125	dead at 54 d.
	29396	275	dead at 65 d.
	$\bar{X}$	220	209
T	29326	186	190
	29333	145	150
	29338	192	196
	29390	225	230
	29392	320	333
	29400	260	dead at 64 d.
	$\bar{X}$	221	218

TABLE 22a

SKIN THICKNESS MEASUREMENTS IN INCHES FOLLOWING INTRACUTANEOUS INJECTION OF MAMMALIAN P.P.D. INTO THE WING WEB OF THE RIGHT WING (R.W.). THE LEFT WING (L.W.) SERVED AS A CONTROL THROUGHOUT - GNOTOBIOTIC

Gn	Initial Thickness		2 hrs.		4 hrs.		8 hrs.	
Chick #	RW	LW	RW	LW	RW	LW	RW	LW
704	.034	.020	.092	.056	.059	.042	.054	.049
769	.032	.042	.053	.056	.054	.050	.051	.036
751	.038	.020	.052	.051	.044	.042	.026	.032
791(M)	.042	.039	.059	.052	.065	.064	.057	.059
702(M)	.030	.035	.051	.050	.056	.034	.041	.030
732(M)	.038	.047	.070	.055	.061	.048	.059	.056
703	.030	.041	.072	.054	.056	.042	.042	.038
763	.021	.029	.032	.035	.030	.031	.031	.025
799(M)	.031	.040	.042	.030	.041	.025	.030	.033
719	.032	.045	.082	.040	.067	.048	.055	.054
748(M)	.028	.026	.093	.046	.095	.040	.043	.042
728	.044	.041	.068	.052	.052	.050	.038	.050
773	.036	.025	.070	.027	.052	.044	.040	.027
722	.040	.045	.086	.044	.057	.055	.050	.048

	12 hrs.		24 hrs.		48 hrs.		72 hrs.	
	RW	LW	RW	LW	RW	LW	RW	LW
704	.063	.058	.061	.054	.073	.045	.075	.056
769	.044	.049	.055	.038	.062	.039	.058	.045
751	.035	.043	.022	.041	.046	.043	.050	.040
791(M)	.068	.045	.070	.057	.049	.050	.040	.047
702(M)	.044	.042	.056	.051	.037	.039	.034	.032
732(M)	.056	.045	.053	.050	.052	.047	.042	.042
703	.045	.049	.046	.036	.060	.046	.043	.035
763	.034	.042	.033	.030	.035	.025	.030	.023
799(M)	.035	.033	.035	.030	.036	.034	.042	.050
719	.063	.058	.055	.055	.051	.045	.053	.055
748(M)	.044	.041	.049	.050	.044	.046	.039	.050
728	.034	.054	.044	.050	.037	.037	.027	.034
773	.039	.036	.040	.040	.034	.037	.041	.051
722	.057	.059	.053	.044	.049	.051	.051	.051

TABLE 22b

SKIN THICKNESS MEASUREMENTS IN INCHES FOLLOWING INTRACUTANEOUS INJECTION OF MAMMALIAN P.P.D. INTO THE WING WEB OF THE RIGHT WING (R.W.). THE LEFT WING (L.W.) SERVED AS A CONTROL THROUGHOUT - CONVENTIONAL

C	Initial Thickness		2 hrs.		4 hrs.		8 hrs.	
Chick #	RW	LW	RW	LW	RW	LW	RW	LW
550	.026	.029	.053	.048	.046	.047	.050	.042
518	.029	.028	.049	.034	.041	.042	.035	.032
796	.029	.015	.055	.034	.042	.019	.050	.025
738	.025	.021	.037	.035	.042	.039	.042	.027
775(M)	.030	.035	.050	.037	.040	.038	.044	.041
715(M)	.025	.026	.064	.032	.040	.039	.053	.037
730	.034	.032	.061	.031	.051	.050	.047	.036
729	.031	.028	.046	.025	.035	.035	.050	.040
779(M)	.029	.028	.095	.039	.083	.037	.073	.033
594	.043	.038	.057	.038	.057	.037	ND	
596	.043	.035	.051	.045	.057	.042	ND	
755	.034	.038	.043	.032	.037	.043	ND	
564	.039	.040	.054	.052	.054	.048	ND	
588	.033	.037	.045	.043	.059	.047	ND	
780(M)	.026	.031	.038	.034	.042	.023	ND	
739	.038	.026	.042	.035	.046	.036	ND	
29767/68	.035	.044	.043	.030	.043	.034	ND	
766	.032	.030	.051	.030	.042	.040	ND	

TABLE 22b (CONTINUED)

C	12 hrs.		24 hrs.		48 hrs.		72 hrs.	
Chick #	RW	LW	RW	LW	RW	LW	RW	LW
550	ND		.053	.035	.046	.035	.039	.035
518	ND		.037	.036	.043	.037	.038	.035
796	ND		.037	.031	.043	.038	.026	.028
738	ND		.039	.025	.040	.025	.036	.029
775(M)	ND		.077	.048	.052	.042	.054	.040
715(M)	ND		.062	.044	.048	.050	.044	.044
730	ND		.046	.044	.034	.042	.050	.048
729	ND		.032	.039	.043	.038	.035	.030
779(M)	ND		.070	.039	.091	.046	.061	.030
594	ND		.059	.040	.051	.038	ND	
596	ND		.060	.032	.048	.031	ND	
755	ND		.042	.030	.036	.038	ND	
564	ND		.056	.042	.045	.042	ND	
588	ND		.043	.048	.038	.035	ND	
780(M)	ND		.060	.034	.038	.041	ND	
739	ND		.051	.031	.046	.040	ND	
29767/68	ND		.036	.047	.057	.042	ND	
766	ND		.040	.035	.048	.039	ND	

TABLE 23

SKIN THICKNESS MEASUREMENTS IN INCHES FOLLOWING INTRACUTANEOUS INJECTION OF CRUDE BCG INTO THE WEB OF THE RIGHT WING

Gn:	Initial Thickness		5 hrs.		24 hrs.		48 hrs.		72 hrs.	
Chick #	RW	LW	RW	LW	RW	LW	RW	LW	RW	LW
791(M)	.045	.034	.037	.033	.039	.039	.041	.027	.048	.026
702(M)	.030	.028	.033	.029	.028	.022	.027	.026	.041	.018
732(M)	.031	.026	.037	.029	.042	.027	.039	.032	.052	.035
704	.039	.035	.042	.034	.038	.042	.040	.026	.047	.034
769	.030	.031	.038	.038	.031	.037	.040	.034	.041	.035
751	.032	.035	.032	.043	.042	.037	.038	.040	.040	.031
703	.043	.033	.033	.036	.040	.035	.059	.036	.044	.044
763	.021	.025	.025	.020	.023	.022	.033	.021	.040	.019
722	.035	.043	.043	.042	.038	.039	.036	.048	.063	.054

TABLE 23 (CONTINUED)

C:	Initial Thickness		5 hrs.		24 hrs.		48 hrs.		72 hrs.	
Chick #	RW	LW	RW	LW	RW	LW	RW	LW	RW	LW
729	.023	.034	.032	.026	.023	.024	.022	.024	.030	.028
779(M)	.025	.023	.040	.028	.040	.025	.042	.028	.049	.031
715(M)	.037	.029	.034	.027	.036	.029	.042	.040	.057	.036
518	.037	.026	.033	.037	.032	.034	.035	.035	.034	.034
594	.017	.026	.037	.025	.027	.032	.037	.027	.045	.035
596	.022	.028	.030	.027	.042	.030	.045	.032	.042	.021
588	.021	.025	.024	.018	.032	.025	.029	.030	.026	.028
755	.020	.029	.019	.034	.031	.026	.039	.031	.039	.025
780(M)	.023	.021	.041	.037	.045	.038	.042	.026	.050	.037
550	.034	.025	.038	.040	.033	.025	.041	.041	.044	.037
739	.025	.039	.031	.021	.028	.039	.038	.031	.029	.033
738	.033	.026	.039	.022	.029	.022	.036	.028	.044	.033
775(M)	.027	.029	.036	.037	.022	.032	.048	.036	.045	.032
796	.032	.024	.040	.038	.038	.026	.037	.030	.045	.029
766	.023	.030	.028	.034	.032	.026	.032	.031	.032	.020
730	.029	.045	.035	.035	.037	.037	.035	.032	.037	.029

TABLE 24

SIGNIFICANCE LEVELS OF WING WEB THICKNESSES AT THE SIGHT OF THE SKIN TEST AREA

	Init.	2 hr.	4 hr.	8 hr.	12 hr.	24 hr.	48 hr.	72 hr.
Gn: P.P.D. Skin Testing								
RW vs LW	N.S.	$p<0.001$	$p<0.001$	N.S.	N.S.	N.S.	N.S.	N.S.
RW BCG vs RW non-BCG vaccinated	--	--	--	--	N.S.	N.S.	N.S.	N.S.
BCG Skin Testing								
RW vs LW	N.S.	--	$p<0.01$	--	--	N.S.	$p<0.001$	$0.005<$ $p<0.01$
RW BCG vs RW non-BCG vaccinated	--	--	--	--	--	N.S.	N.S.	N.S.

Gn = gnotobiotic
C = conventional
PPD = purified protein derivative
BCG = Bacillus of Calmette and Guérin
RW = right wing (injected - see text)
LW = left wing (control)

TABLE 24 (CONTINUED)

	Init.	2 hr.	4 hr.	8 hr.	12 hr.	24 hr.	48 hr.	72 hr.
C: P.P.D. Skin Testing								
RW vs LW	N.S.	$p<0.001$	N.S.	$0.005<$ $p<0.001$	N.S.	$p<0.001$	$0.01<$ $p<0.05$	$0.025<$ $p<0.05$
RW BCG vs RW non-BCG vaccinated	--	--	--	--	--	$0.001<$ $p<0.005$	$0.01<$ $p<0.05$	$0.005<$ $p<0.01$
BCG Skin Testing								
RW vs LW	N.S.	--	N.S.	--	--	N.S.	$0.005<$ $p<0.01$	$p<0.001$
RW BCG vs RW non-BCG vaccinated	--	--	--	--	--	N.S.	$0.025<$ $p<0.05$	$0.005<$ $p<0.01$

TABLE 25

PLAQUE FORMING CELLS (PFC) PER MILLION SPLEEN CELLS OF GNOTOBIOTIC AND CONVENTIONALLY HOUSED $\underline{B}^2\underline{B}^2$ WHITE LEGHORNS, BURSECTOMIZED (B̸) AND SHAM OPERATED (B) ON HATCHING. THE ASSAY WAS PERFORMED AS A TERMINAL EXPERIMENT AT AGE 5 TO 5½ MONTHS, FIVE DAYS FOLLOWING INJECTION OF A STANDARD DOSE OF 5 x 10^8 SRBC

		PFC/10^6 Spleen Cells					
Gnotobiotic		Test 1	Test 2	Conven.		Test 1	Test 2
B̸(M)	748	626.	636.	B̸	729	1.0	0.5
B̸+C	722	0	0	B̸(M)	755	114.3	100.
	751	0	0		779	49.5	44.2
B̸+C(M)	799	0	0	B̸+C	796	<1.	<1.
	763	0	0	B+C(M)	775	1.5	0.8
	719	4.4	4.4		594	0.3	1.0
U(M)	732	558.	525.	B	739	29,200.	24,133.
B	704	810.	383.		564	3,103.	3,379.
	702	827.	392.	B(M)	780	2,720.	1,840.
B+C	769	1,999.	1,809.		738	3,533.	4,133.
	728	367.	367.	B+C	518	1,789.	2,105.
B+C(M)	791	1,735.	1,578.		730	1,887.	1,962.
	703	1,591.	814.		766	338.	394.
					588	2,061.	1,939.
				B+C(M)	715	1.463.	2,781.
					596	1,000.	1,786.
					550	51.6	32.25

B̸ = Bursectomized
B̸(M) = Bursectomized and vaccinated with BCG (Mycobacteria)
B̸+C = Bursectomized and cyclophosphomide treated
B̸+C(M) = Bursectomized, cyclophosphomide treated and BCG vaccinated
B = sham operated (non-bursectomized)
U = totally untreated at hatching

TABLE 26

BODY WEIGHTS IN GRAMS OF BURSECTOMIZED AND SHAM OPERATED GNOTOBIOTIC AND CONVENTIONALLY HOUSED $\underline{B}^{2}\underline{B}^{2}$ WHITE LEGHORNS, COMPARED WITH SPLEEN WEIGHTS IN MILLIGRAMS

Gnotobiotic:			Body Weight (gm.)	Spleen Weight (mg.)
Ƀ(M)	748	(f)	500.	330.
Ƀ+C	722	(f)	705.	530.
	751	(m)	500.	530.
Ƀ+C(M)	791	(m)	480.	360.
	763	(m)	540.	430.
	719	(f)	460.	260.
U(M)	732	(f)	630.	840.
B	704	(f)	585.	1200.
	702	(m)	680.	710.
B+C	769	(f)	585.	960.
	728	(m)	620.	590.
B+C(M)	703	(f)	540.	560.
	791	(f)	615.	650.
		Mean $\log_{10}$:	2.7540	2.7493

(f) = female
(m) = male
Ƀ = Bursectomized
Ƀ(M) = Bursectomized and vaccinated with BCG (Mycobacteria)
Ƀ+C = Bursectomized and cyclophosphomide treated
Ƀ+C(M) = Bursectomized, cyclophosphomide treated and BCG vaccinated
B = sham operated (non-bursectomized)
U = totally untreated at hatching

TABLE 26 (CONTINUED)

Conventional:			Body Weight (gm.)	Spleen Weight (mg.)
B̸	729	(f)	525.	580.
B̸(M)	755	(m)	680.	420.
	779	(m)	575.	340.
B̸+C	796	(f)	470.	390.
B̸+C(M)	775	(f)	600.	1400.
	594	(f)	525.	710.
B	739	(m)	520.	370.
	564	(m)	578.	520.
B(M)	780	(m)	455.	440.
	738	(m)	530.	500.
B+C	518	(m)	565.	370.
	730	(f)	570.	760.
	766	(m)	805.	590.
	588	(f)	435.	530.
B+C(M)	715	(m)	645.	560.
	596	(f)	478.	540.
	550	(m)	615.	480.
		Mean $\log_{10}$:	2.7455	2.7199

TABLE 27

TITRES OF ANTIBODY AGAINST SHEEP RED BLOOD CELLS (SRBC) IN BURSECTOMIZED AND NON-BURSECTOMIZED GNOTOBIOTIC AND CONVENTIONALLY HOUSED $\underline{B}^2\underline{B}^2$ WHITE LEGHORNS (RECIPROCALS OF TITRES GIVEN). LEGEND FOR ANIMAL TREATMENTS AS IN TABLE 25.

Individual		Pre-SRBC	5 d. Post-SRBC
Gn:			
Ƀ(M)	748	-	-
Ƀ+C	751	-	-
	722	-	-
Ƀ+C(M)	799	-	-
	719	-	-
	763	-	-
B	704	-	128
B(M)	702	-	128
B+C	769	-	128+
B+C(M)	791	-	64
	728	-	64
	703	-	128
U(M)	732	4	128

TABLE 27 (CONTINUED)

Individual		Pre-SRBC	5 d. Post-SRBC
C:			
B̸	729	-	-
	755	-	-
B̸(M)	779	-	-
	775	-	-
B̸+C	796	-	-
B̸+C(M)	594	-	-
B	739	-	64
	564	T	32
B(M)	780	-	128
	738	-	128
B+C	518	-	32
	766	-	32
	730	-	128
	588	-	64
B+C(M)	715	-	32
	596	-	128+
	550	-	-

TABLE 28

ACQUISITION OF ANTI-O BY SHAM OPERATED AND THREE BURSECTOMIZED GNOTOBIOTIC $\underline{B}^2\underline{B}^2$ LEGHORNS.
NOTE: NO OTHER BURSECTOMIZED ANIMALS DEVELOPED ANY ANTIBODY.

		Age						5 to 5.5 mo.	
Chick #/Treatment		1 mo.	1.5 mo.	2 mo.	2.5 mo.	3 mo.	4.5 mo.	Pre-SRBC	Post-SRBC
B(M)	748	0.0	0.0	1.0	N.D.	N.D.	N.D.	0.0	0.0
B+C	722	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0
	751	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
B	704	0.0	0.0	0.0	0.0	0.0	0.0	5.0	7.0
	709	0.0(dead)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
B+C	769	0.0	0.0	0.0	N.D.	0.0	0.0	0.0	4.0
	773	1.0	N.D.	1.0	0.0(dead)	N.D.	N.D.	N.D.	N.D.
B(M)	702	0.0	0.0	0.0	N.D.	0.0	0.0	0.0	4.0
B+C(M)	791	0.0	0.0	0.0	N.D.	0.0	0.0	0.0	4.0
	728	1.0	0.0	0.0	0.0	0.0	N.D.	0.0	5.0
	703	2.0	0.0	0.0	N.D.	0.0	0.0	0.0	5.0
U(M)	732	0.0	0.0	0.0	N.D.	0.0	1.0	1.0	5.0

N.D. = Not done.

TABLE 29

ACQUISITION OF ANTI-A_1 (Rh-NEGATIVE) BY SHAM OPERATED CONVENTIONAL $\underline{B}^2\underline{B}^2$ LEGHORNS. THEIR BURSECTOMIZED COUNTERPARTS DEVELOPED NO ANTIBODY.

Chick #/Treatment		Age						5 to 5.5 mo.	
		1 mo.	1.5 mo.	2 mo.	2.5 mo.	3 mo.	4.5 mo.	Pre-SRBC	Post-SRBC
B	739	N.D.	0.0	N.D.	0.0	0.0	0.0	0.0	2.0
B+C	518	N.D.	0.0	0.0	0.0	N.D.	0.0	0.0	1.0
	766	N.D.	0.0	0.0	N.D.	0.0	0.0	0.0	0.0
	730	N.D.	0.0	0.0	0.0	1.0	0.0	0.0	0.0
	588	0.0	N.D.	0.0	N.D.	N.D.	0.0	0.0	0.0
B(M)	780	N.D.	0.0	0.0	2.0	1.0	0.0	0.0	2.0
	738	N.D.	0.0	0.0	0.0	N.D.	2.0	0.0	0.0
B+C(M)	715	N.D.	0.0	4.0	4.0	N.D.	0.0	0.0	1.0
	596	0.0	N.D.	0.0	N.D.	N.D.	0.0	0.0	2.0
	550	N.D.	0.0	0.0	0.0	N.D.	0.0	0.0	0.0

N.D. = Not done.

TABLE 30

ACQUISITION OF ANTI-B (Rh-NEGATIVE) BY SHAM OPERATED CONVENTIONAL $\underline{B}^{2}\underline{B}^{2}$ LEGHORNS. THEIR BURSECTOMIZED COUNTERPARTS DEVELOPED NO ANTIBODY.

Chick #/Treatment		Age						5 to 5.5 mo.	
		1 mo.	1.5 mo.	2 mo.	2.5 mo.	3 mo.	4.5 mo.	Pre-SRBC	Post-SRBC
B	739	N.D.	0.0	N.D.	0.0	0.0	3.0	0.0	3.0
B+C	518	N.D.	0.0	0.0	0.0	N.D.	0.0	0.0	2.0
	766	N.D.	0.0	0.0	N.D.	0.0	2.0	0.0	0.0
	730	N.D.	0.0	2.0	3.0	3.0	0.0	2.0	1.5
	588	0.0	N.D.	0.0	N.D.	3.0	3.0	4.0	4.0
B(M)	780	N.D.	0.0	0.0	5.0	2.0	2.0	4.0	6.0
	738	N.D.	0.0	0.0	0.0	N.D.	3.0	3.0	2.0
B+C(M)	715	N.D.	0.0	0.0	0.0	N.D.	0.0	0.0	1.0
	596	0.0	N.D.	0.0	N.D.	N.D.	4.0	7.0	8.0
	550	N.D.	0.0	0.0	0.0	N.D.	0.0	0.0	0.0

N.D. = Not done.

TABLE 31

ACQUISITION OF ANTI-O BY SHAM OPERATED AND ONE BURSECTOMIZED CONVENTIONAL $\underline{B}^2\underline{B}^2$ LEGHORNS. NO OTHER BURSECTOMIZED ANIMALS DEVELOPED ANY ANTIBODY.

Chick #/Treatment		Age						5 to 5.5 mo.	
		1 mo.	1.5 mo.	2 mo.	2.5 mo.	3 mo.	4.5 mo.	Pre-SRBC	Post-SRBC
B+C(M)	775	N.D.	1.0	0.0	0.0	N.D.	0.0	0.0	0.0
B	739	N.D.	0.0	N.D.	0.0	0.0	3.0	3.0	5.0
B+C	518	N.D.	2.0	0.0	1.0	N.D.	1.0	1.0	4.0
	766	N.D.	0.0	0.0	N.D.	0.0	2.0	0.0	2.0
	730	N.D.	0.0	3.0	5.0	3.0	1.0	2.0	5.0
	588	0.0	N.D.	1.0	N.D.	3.0	3.0	5.0	7.0
B(M)	780	N.D.	0.0	0.0	3.0	2.0	5.0	5.0	7.0
	738	N.D.	0.0	0.0	0.0	N.D.	4.0	3.0	5.0
B+C(M)	715	N.D.	0.0	4.0	5.0	N.D.	3.0	0.0	3.0
	596	0.0	N.D.	4.0	N.D.	N.D.	6.0	7.0	8.0
	550	N.D.	0.0	0.0	0.0	N.D.	0.0	0.0	0.0

N.D. = Not done.

FIGURE 1a: Flexible plastic isolation chamber for housing germfree and gnotobiotic animals, front view.

- a. Sleeves with attached gloves.
- b. Outlet trap with sump.
- c. Inlet filtering system with hose connecting blower-motor.

FIGURE 1b: Flexible plastic isolation chamber, back view, showing stainless steel port for moving supplies in and out of chamber.

- a. Outside cap with nipples for spraying interior of port with peracetic acid.
- b. Inside cap.
- c. Inlet filtering system.

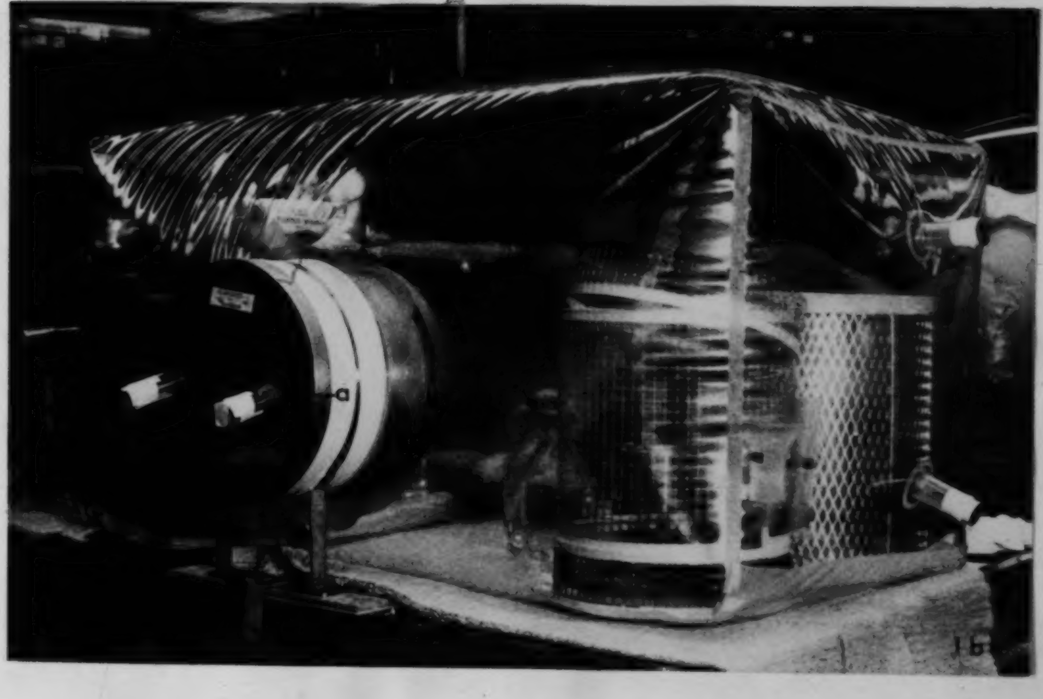

a
1b

FIGURE 2a: Solid hatching isolator, front view with glass plate window.

a. Gloves.

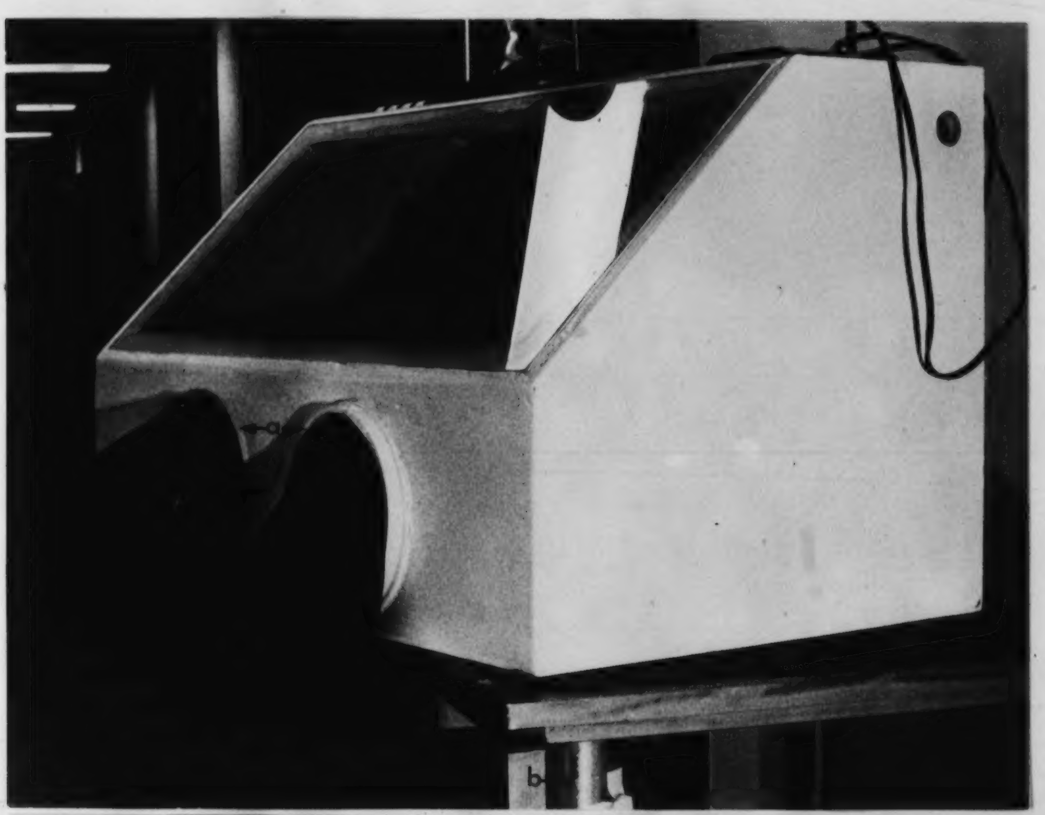
b. Cylinder for drawing eggs into isolator.

FIGURE 2b: Solid hatching isolator, end view.

a. 9" stainless steel port.

b. Outlet filter unit.

c. Inlet filter unit with attached blower-motor.



2 b

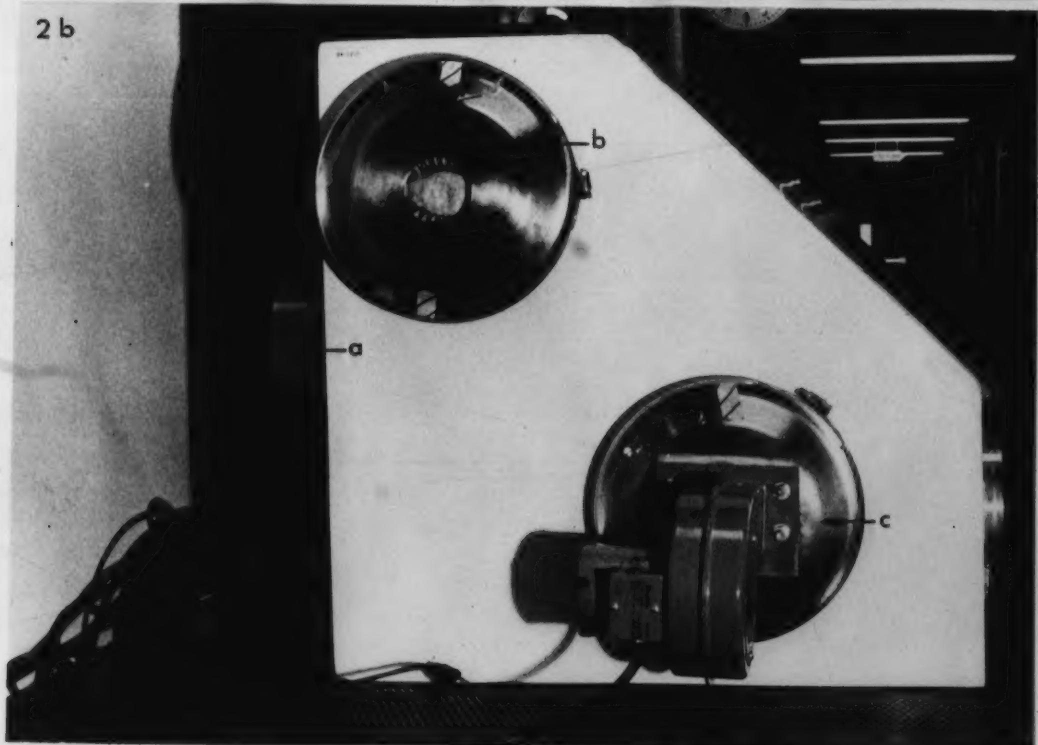


FIGURE 3: Flexible plastic surgical isolator showing the surgical extension with attached gloves. The upper surface of the extension was composed of a special see-through plastic which permitted carrying out fine surgery.

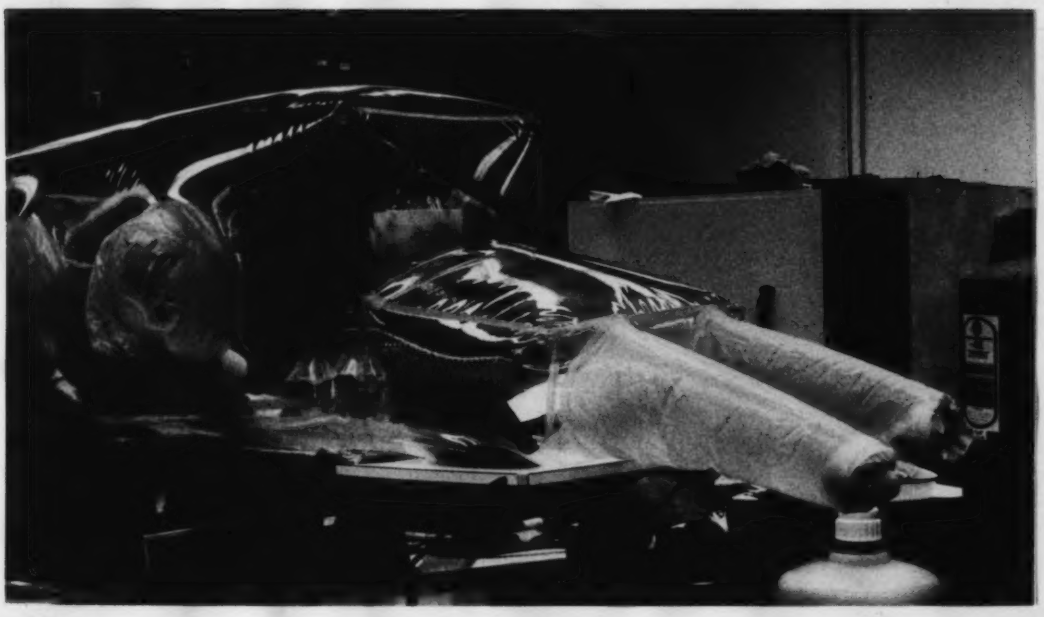


FIGURE 4: Graft-versus-host (GVH) competence of gnotobiotic (Gn) and conventional (C) $\underline{B}^2\underline{B}^2$ chickens on a tryptophane-deficient diet and conventional on a conventional diet (C + C) with age as measured by the chorioallantoic membrane pock (CAMP) test and recipient embryo spleen weight (MES).

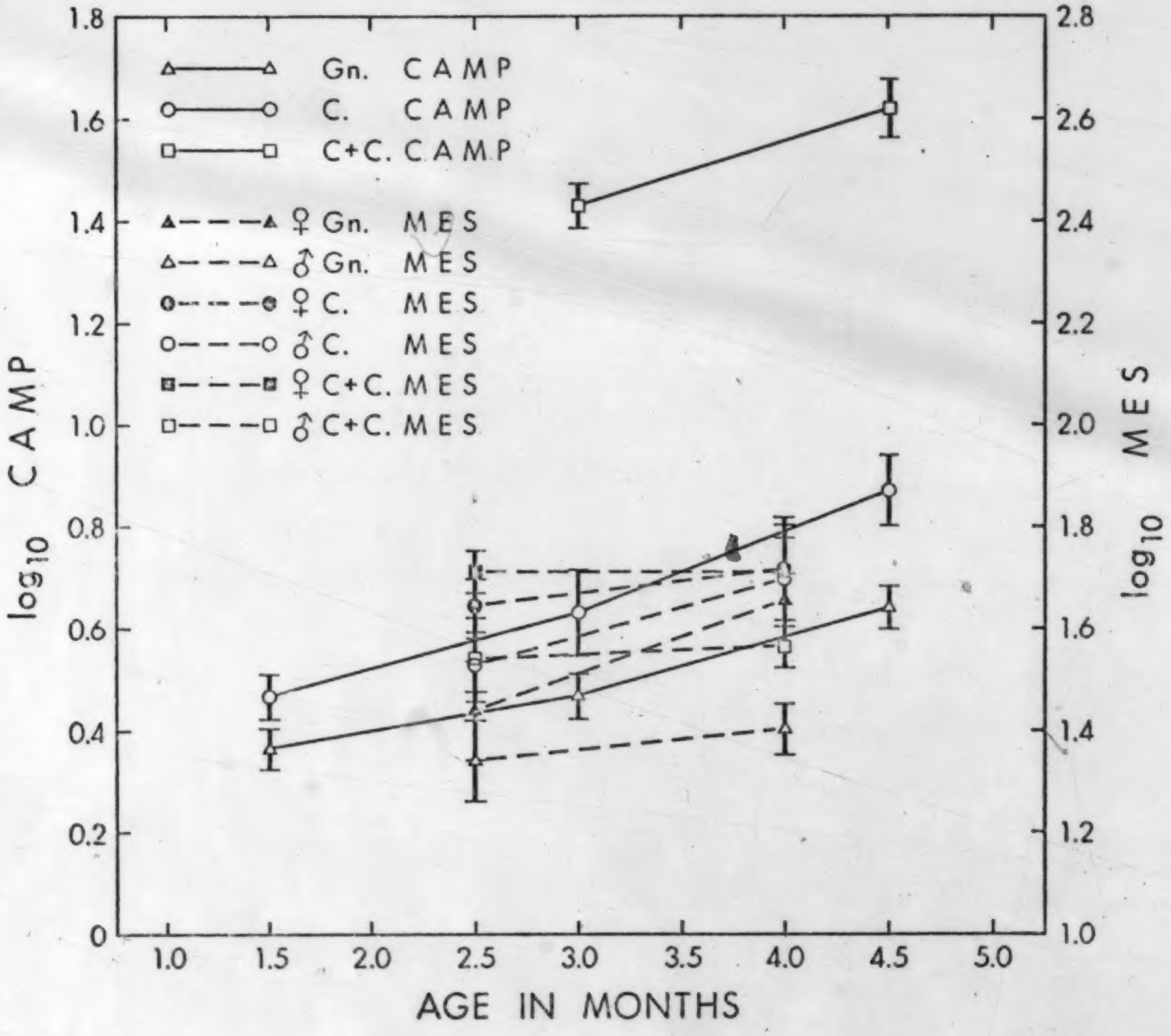

Gn. CAMP
C. CAMP
C+C. CAMP
♀ Gn. MES
♂ Gn. MES
♀ C. MES
♂ C. MES
♀ C+C. MES
♂ C+C. MES
log10 CAMP
log10 MES
AGE IN MONTHS
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0

FIGURE 5: Acquisition of antibody to human blood groups by conventionally housed $\underline{B}^{2}\underline{B}^{2}$ chickens with age. A given point represents the means of all endpoints for that age.

FIGURE 6: Body weight of gnotobiotic (Gn) and conventional (C) $\underline{B}^{2}\underline{B}^{2}$ chickens on a tryptophane-deficient diet and conventional on a conventional diet (C + C) with age.

Figure 5

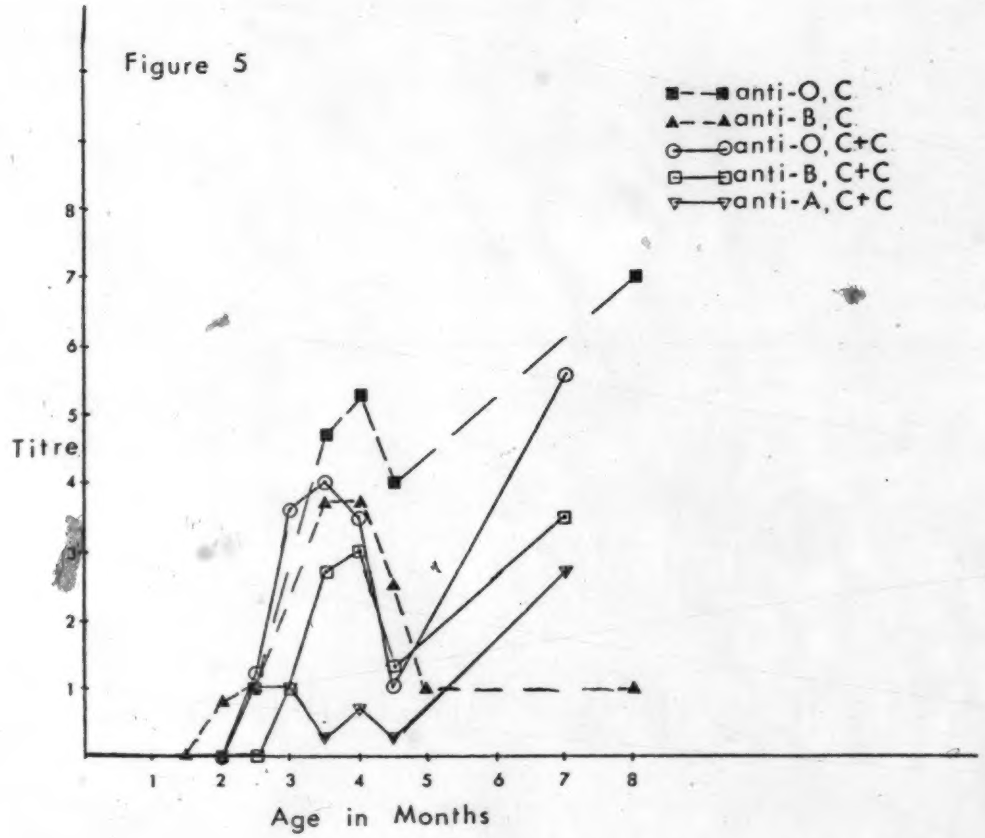


Figure 6

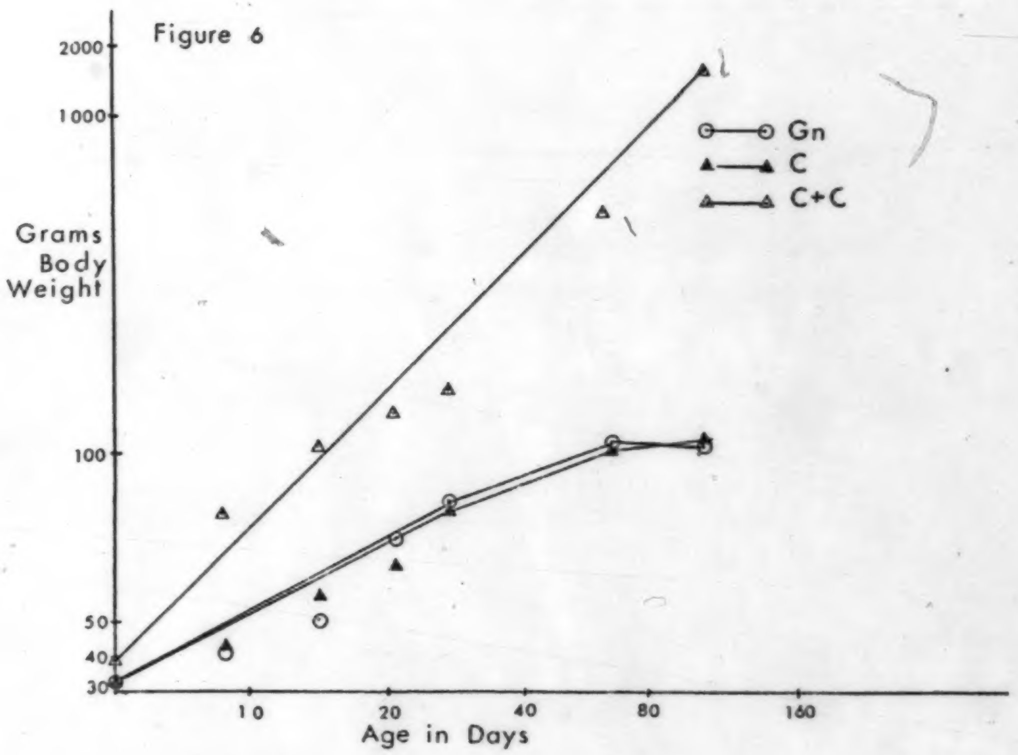


FIGURE 7: Survival of gnotobiotic and conventional $\underline{B}^2\underline{B}^2$ chickens on a tryptophane-deficient diet and conventional on a conventional diet.

Gn = Gnotobiotic.

C = Conventional on a tryptophane-deficient diet.

C + C = Conventional on a conventional diet.

Figure 7

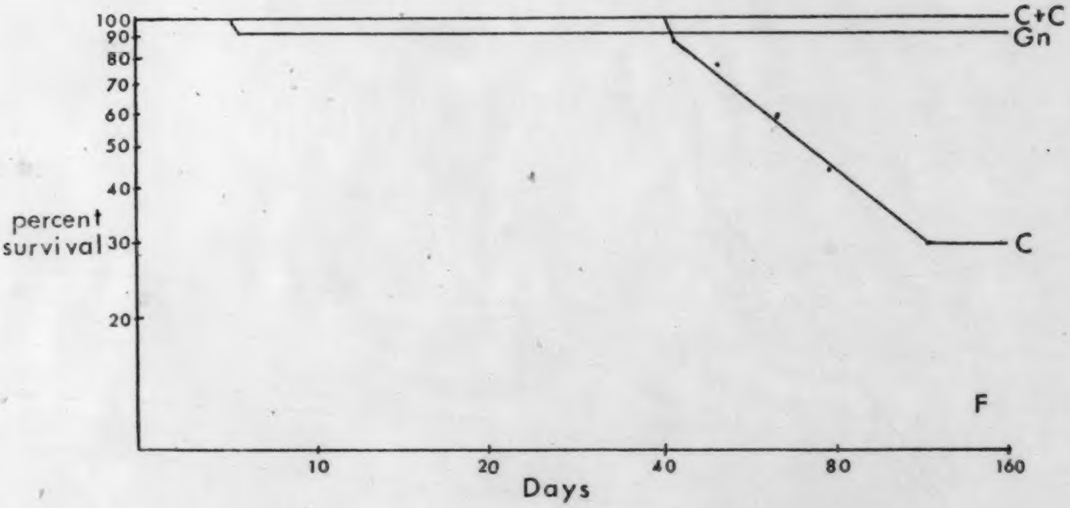

100
90
80
70
60
50
40
percent survival
30
20
C+C
Gn
C
F
10
20
40
80
160
Days

FIGURE 8: Antibody detectable with human A_1, B and O cells plotted as the mean of the titres of ganglionectomized and sham operated chickens in the unadsorbed plasma.

Figure 8

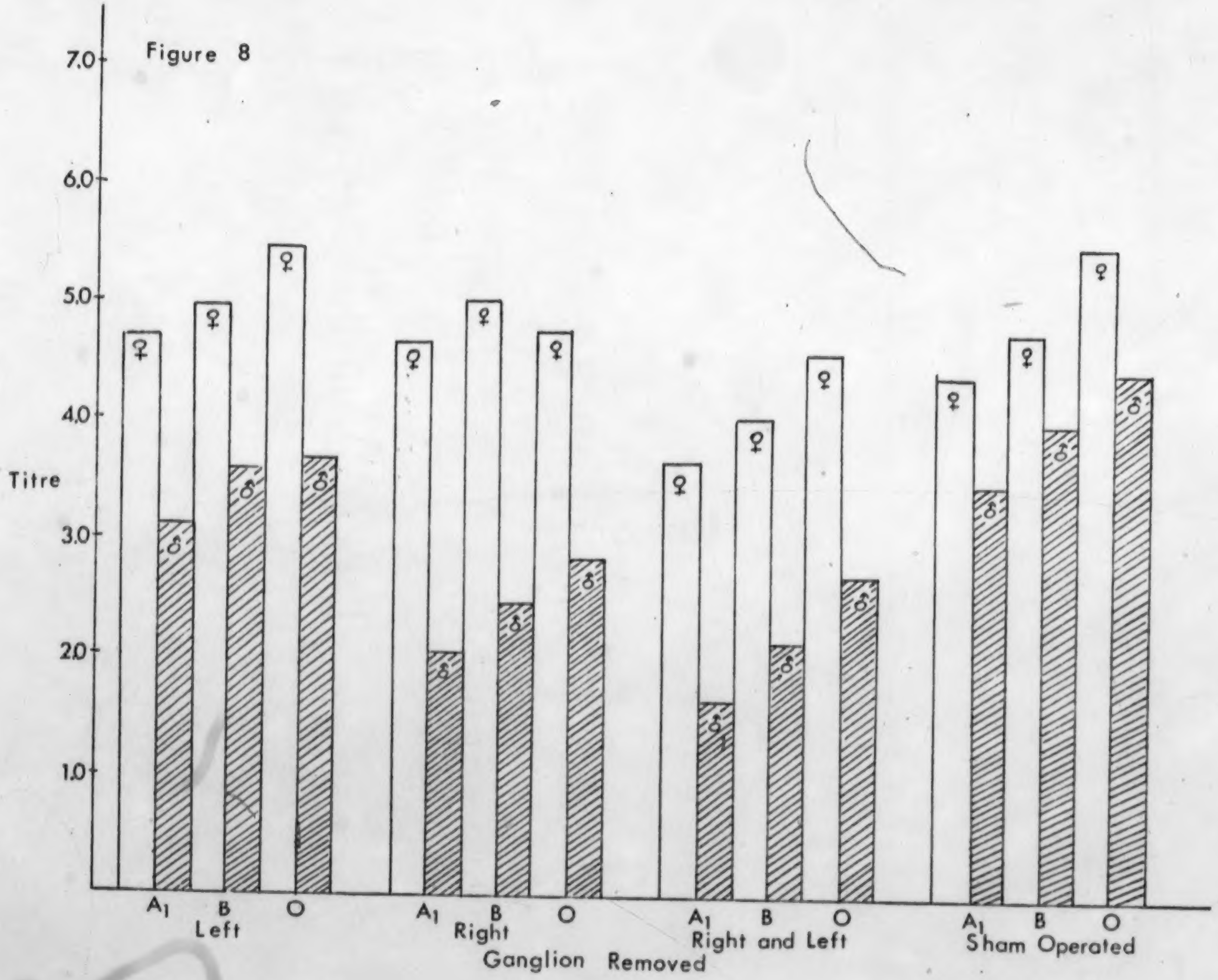

7.0
6.0
5.0
4.0
3.0
2.0
1.0
Titre
♀
♂
A1
B
O
Left
Right
Right and Left
Sham Operated
Ganglion Removed

FIGURE 9: Antibody against human A_1 and B, Rh-negative cells plotted as the mean of the titres of ganglionectomized and sham operated chickens in the adsorbed (four times with O, Rh-positive human red cells) plasma.

Figure 9

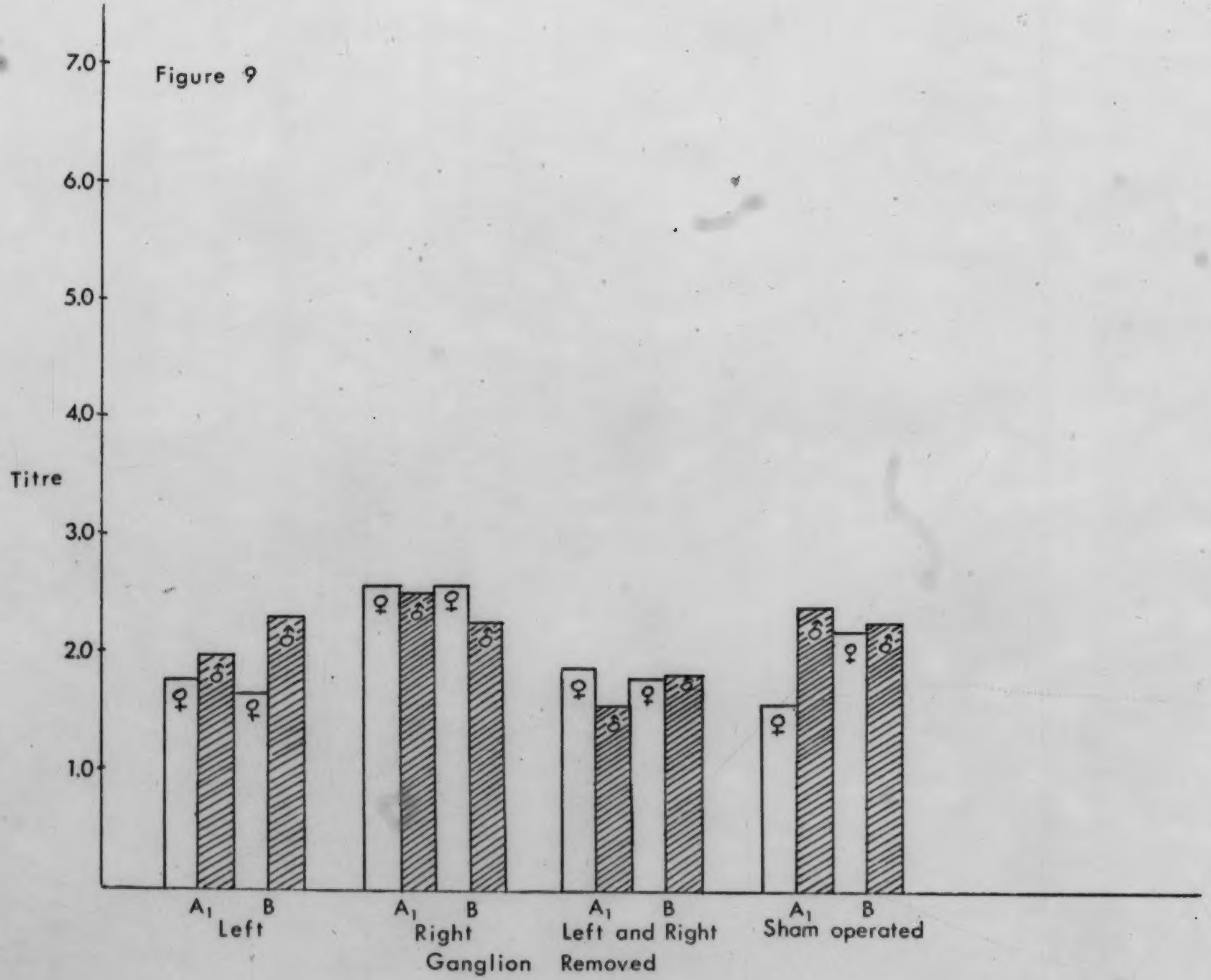

Titre
7.0
6.0
5.0
4.0
3.0
2.0
1.0
♀
♂
A₁
B
Left
Right
Left and Right
Sham operated
Ganglion Removed

FIGURE 10: Correlation of conventional (Conv.) CAMP with gnotobiotic (Gn) CAMP. All three genotypes, $\underline{B}^{2}\underline{B}^{2}$, $\underline{B}^{2}\underline{B}^{14}$, and $\underline{B}^{14}\underline{B}^{14}$ are included at ages 3 to 7 days, 3 to 4 weeks, and 3 months. See text, as well as Table 8. All animals included in this experiment were placed on tryptophane-deficient diet at age 3½ weeks.

FIGURE 11: Mean $\log_{10}$ CAMP of $\underline{B}^{2}\underline{B}^{2}$, $\underline{B}^{2}\underline{B}^{14}$, and $\underline{B}^{14}\underline{B}^{14}$ donors at ages 3 to 7 days, 3 to 4 weeks, 3 months and 6 months. It should be noted that the source and quality of host embryos were different at 6 months of age as opposed to those used at earlier ages.

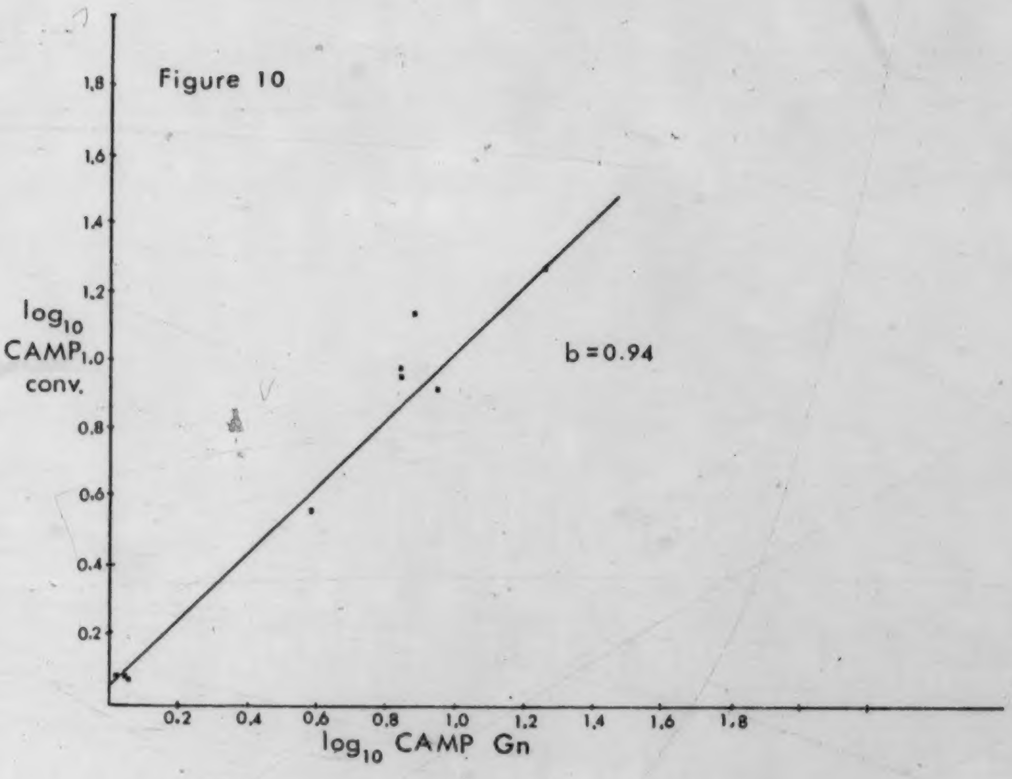


Figure 10

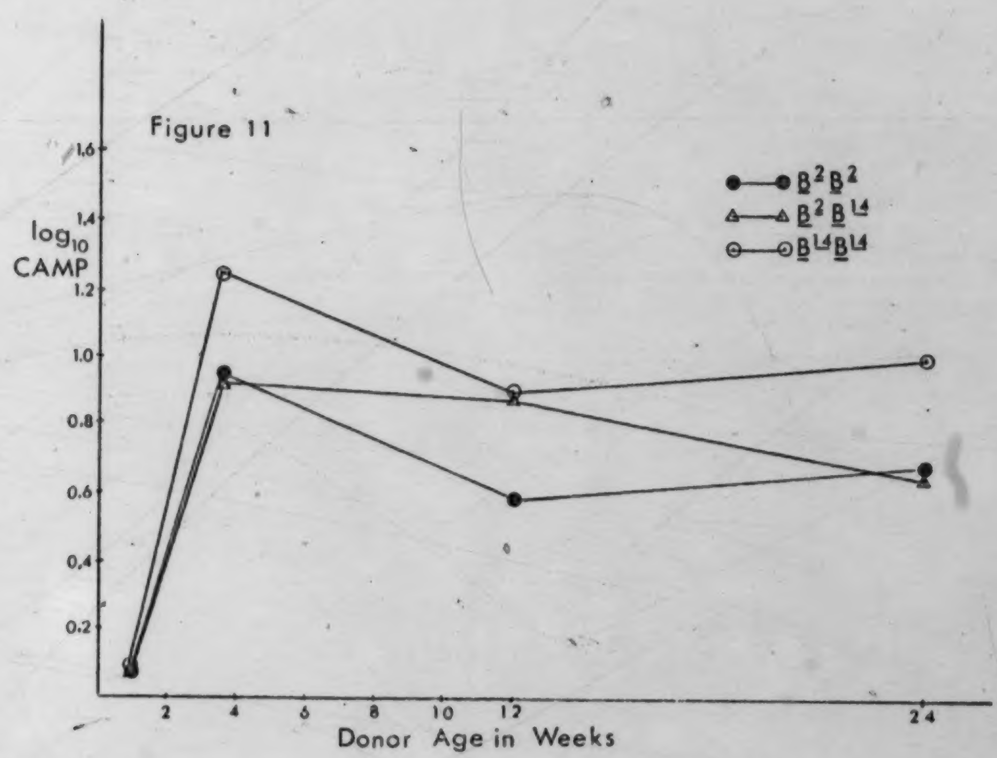


Figure 11

FIGURE 12: Replicate mean $\log_{10}$ CAMP and MES of Gn and Conv. $\underline{B}^{2}\underline{B}^{2}$, $\underline{B}^{2}\underline{B}^{14}$ and $\underline{B}^{14}\underline{B}^{14}$ donors on a tryptophane-deficient diet.

Figure 12

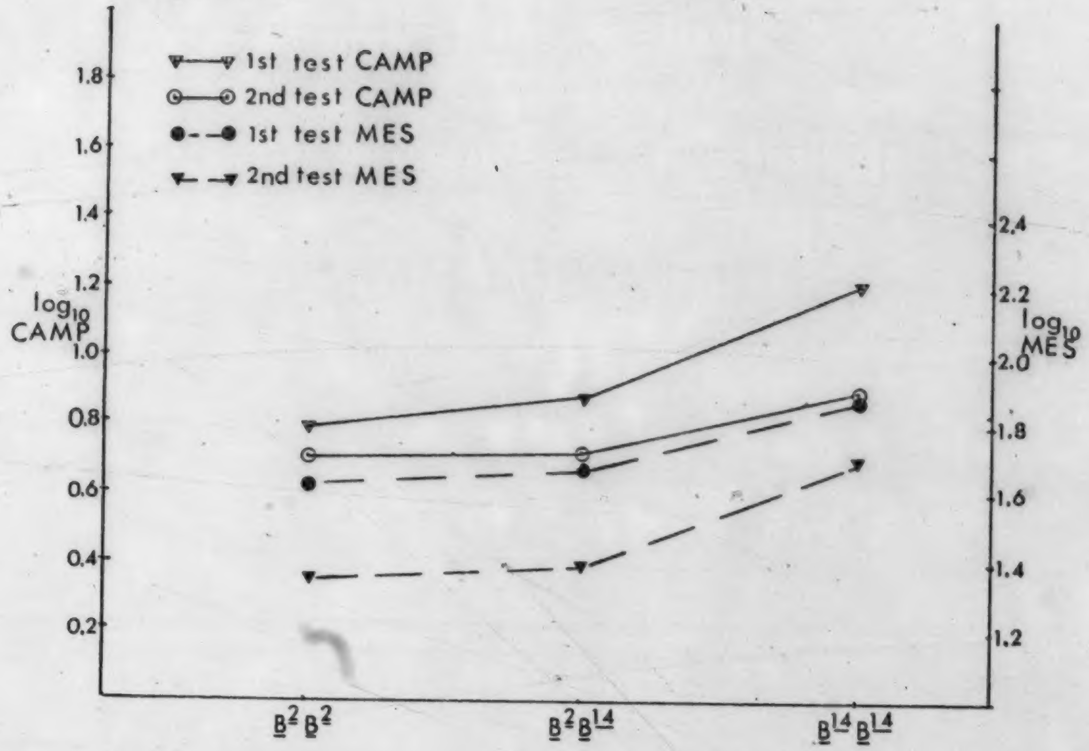

1st test CAMP
2nd test CAMP
1st test MES
2nd test MES
log₁₀ CAMP
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
log₁₀ MES
2.4
2.2
2.0
1.8
1.6
1.4
1.2
B²B²
B²B¹⁴
B¹⁴B¹⁴

FIGURE 13: GVH-CAMP and GVH-MES including recipient embryo sex differences at 6 months of age for $\underline{B}^{2}\underline{B}^{2}$, $\underline{B}^{2}\underline{B}^{14}$ and $\underline{B}^{14}\underline{B}^{14}$ chickens.

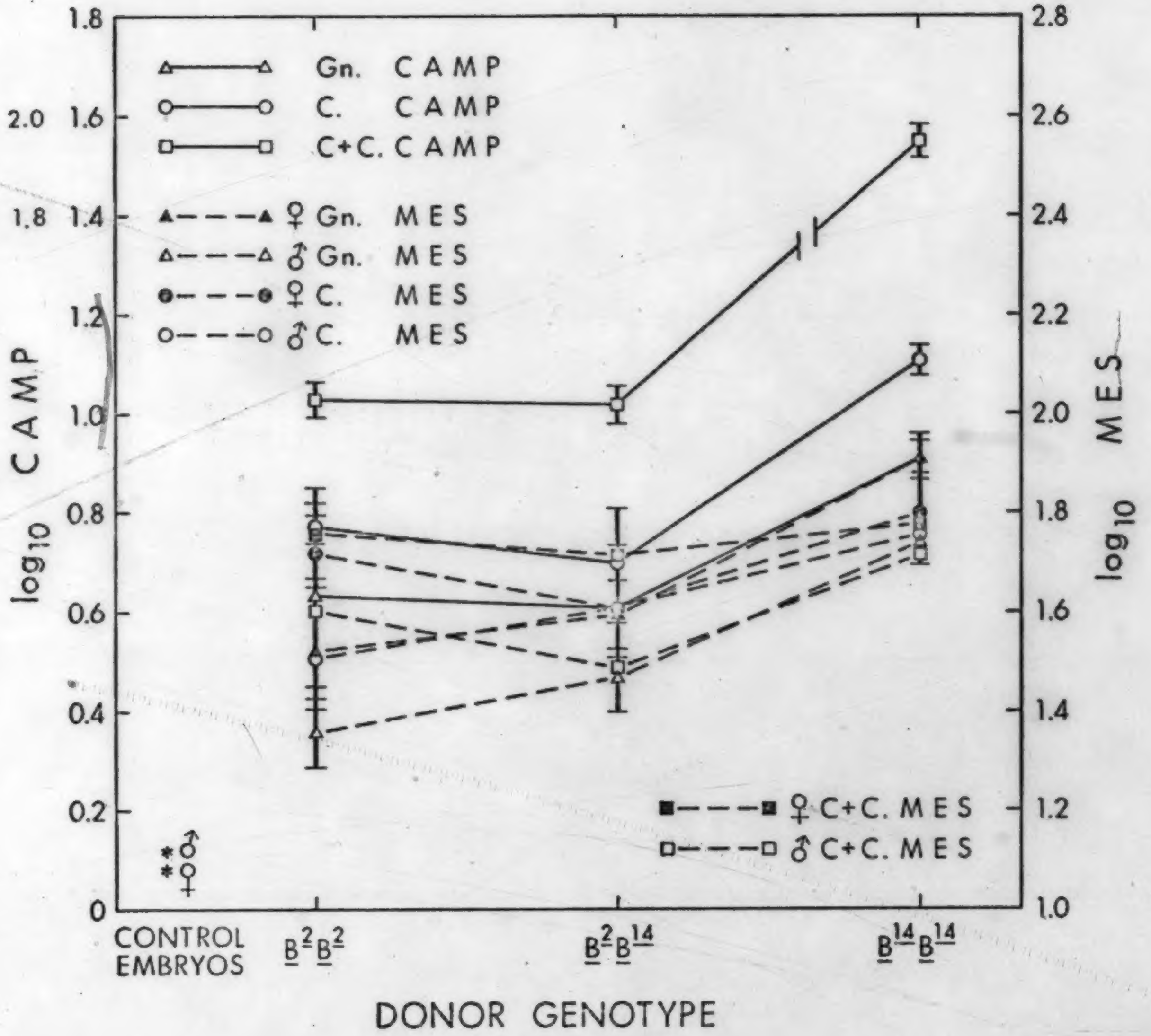

1.8
2.0 1.6
1.8 1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
log₁₀ C A M P
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
log₁₀ M E S
Gn. C A M P
C. C A M P
C+C. C A M P
♀ Gn. M E S
♂ Gn. M E S
♀ C. M E S
♂ C. M E S
♀ C+C. M E S
♂ C+C. M E S
*♂
*♀
CONTROL EMBRYOS
B²B²
B²B¹⁴
B¹⁴B¹⁴
DONOR GENOTYPE

FIGURE 14: GVH-CAMP at 6 months for $\underline{B}^2\underline{B}^2$, $\underline{B}^2\underline{B}^{14}$ and $\underline{B}^{14}\underline{B}^{14}$ donors according to donor means.

FIGURE 15: Percentage response of $\underline{B}^2\underline{B}^2$, $\underline{B}^2\underline{B}^{14}$ and $\underline{B}^{14}\underline{B}^{14}$ gnotobiotic and conventional chickens on the tryptophane-deficient diet with plasma agglutinins for O cells and for A_1 and B human antigens.

Figure 14

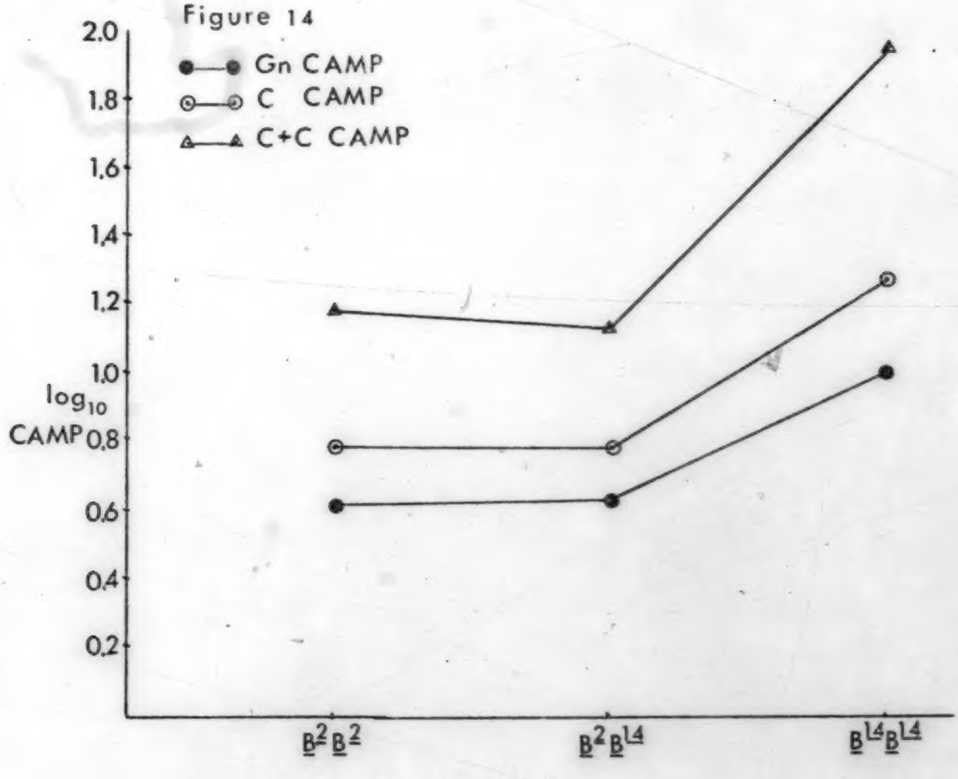


Figure 15

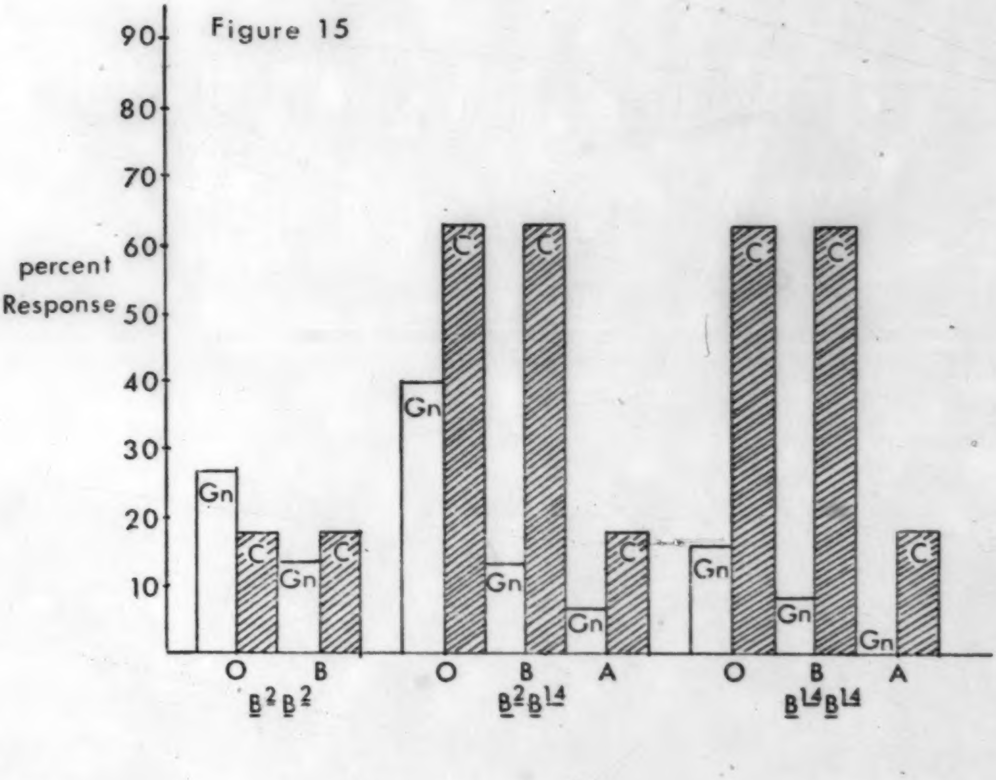


FIGURE 16: Acquisition of anti-O by those responding gnotobiotic (all three genotypes) and conventional $\underline{B^{2}}\underline{B^{2}}$, $\underline{B^{2}}\underline{B^{14}}$ and $\underline{B^{14}}\underline{B^{14}}$ chickens on tryptophane-deficient diet. Each point represents the mean titre at a given age.

FIGURE 17: Acquisition of anti-A_1 (Rh-negative) by conventional $\underline{B^{2}}\underline{B^{2}}$, $\underline{B^{2}}\underline{B^{14}}$ and $\underline{B^{14}}\underline{B^{14}}$ chickens on conventional diet.

Figure 16

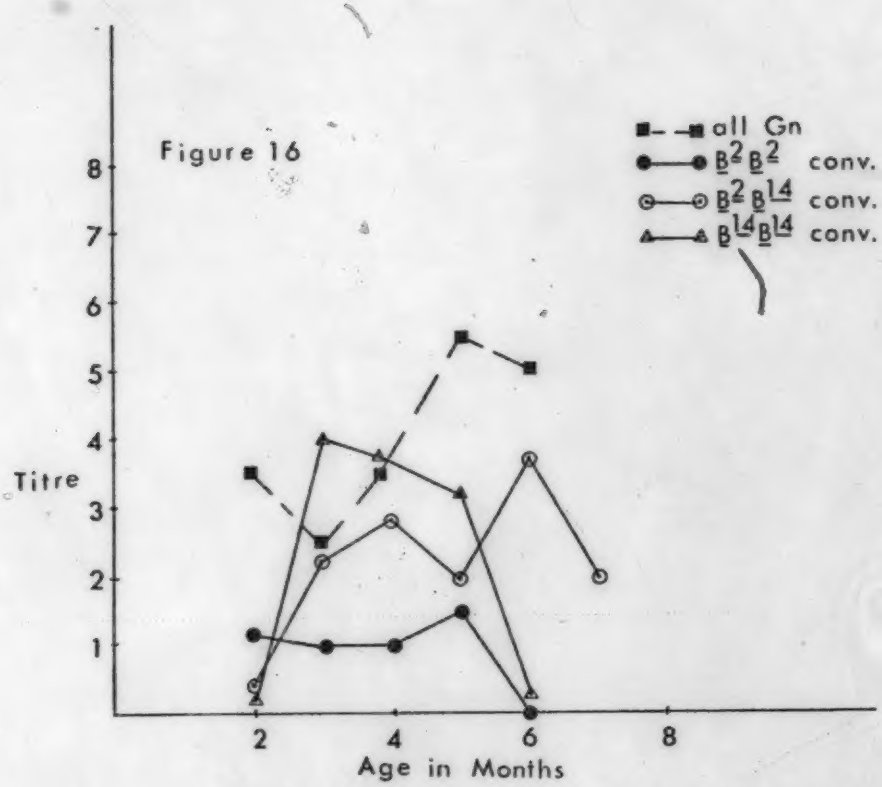


Figure 17

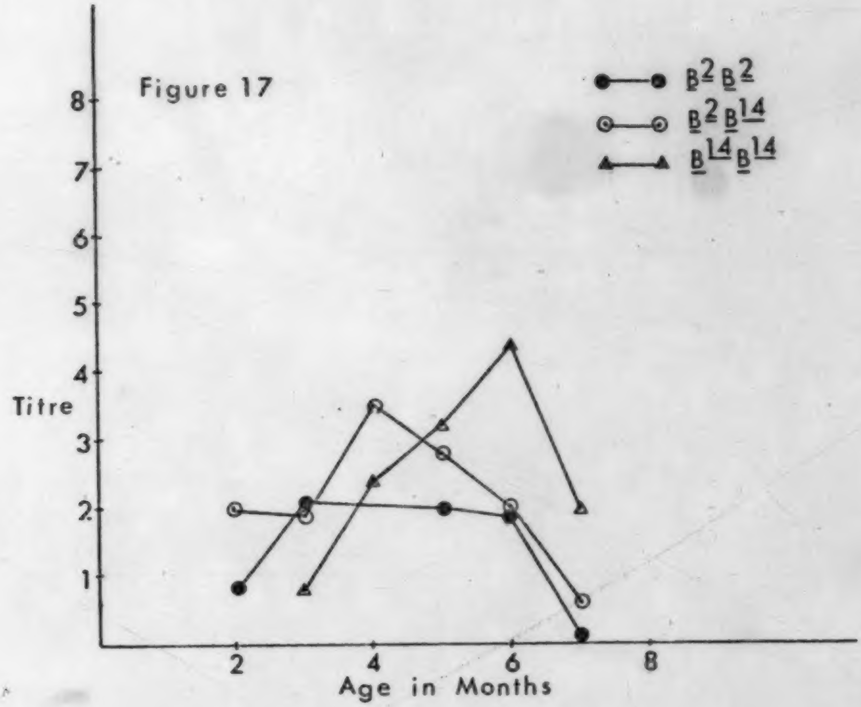


FIGURE 18: Acquisition of anti-B (Rh-negative) by conventional $B^{2}B^{2}$, $B^{2}B^{14}$ and $B^{14}B^{14}$ chickens on conventional diet.

FIGURE 19: Acquisition of anti-O and antibody to any species determinants by conventional $B^{2}B^{2}$, $B^{2}B^{14}$ and $B^{14}B^{14}$ chickens on conventional diet.

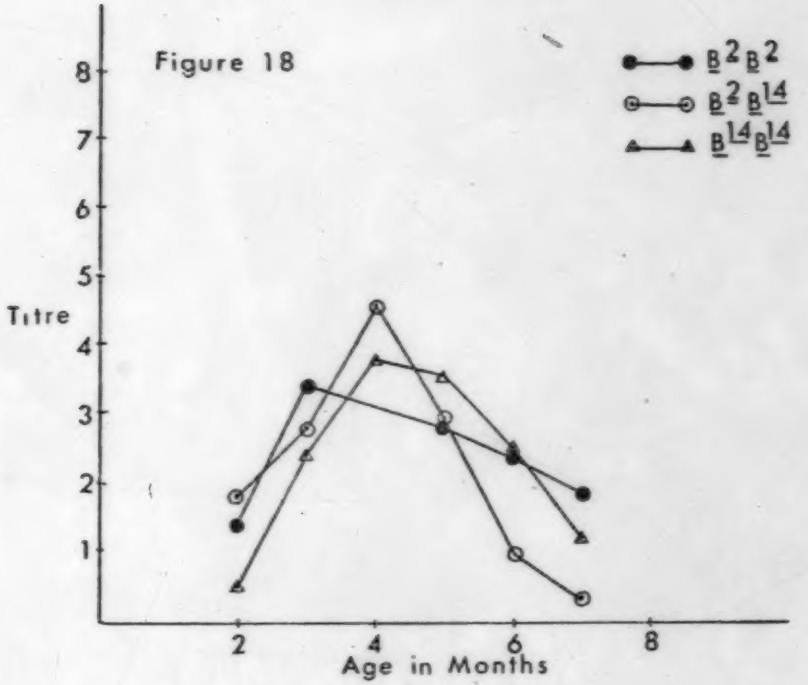


Figure 18

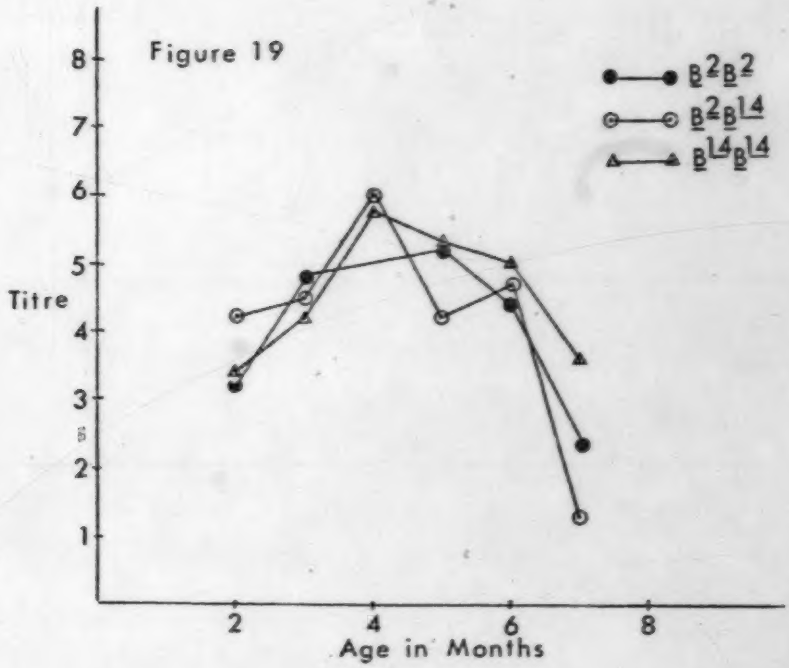


Figure 19

FIGURE 20: Survival of $\underline{B}^{2}\underline{B}^{2}$, $\underline{B}^{2}\underline{B}^{14}$ and $\underline{B}^{14}\underline{B}^{14}$ chickens in the gnotobiotic (Gn) and conventional (C) environments on a tryptophane-deficient diet and in the conventional environment on a conventional diet (C + C).

Figure 20

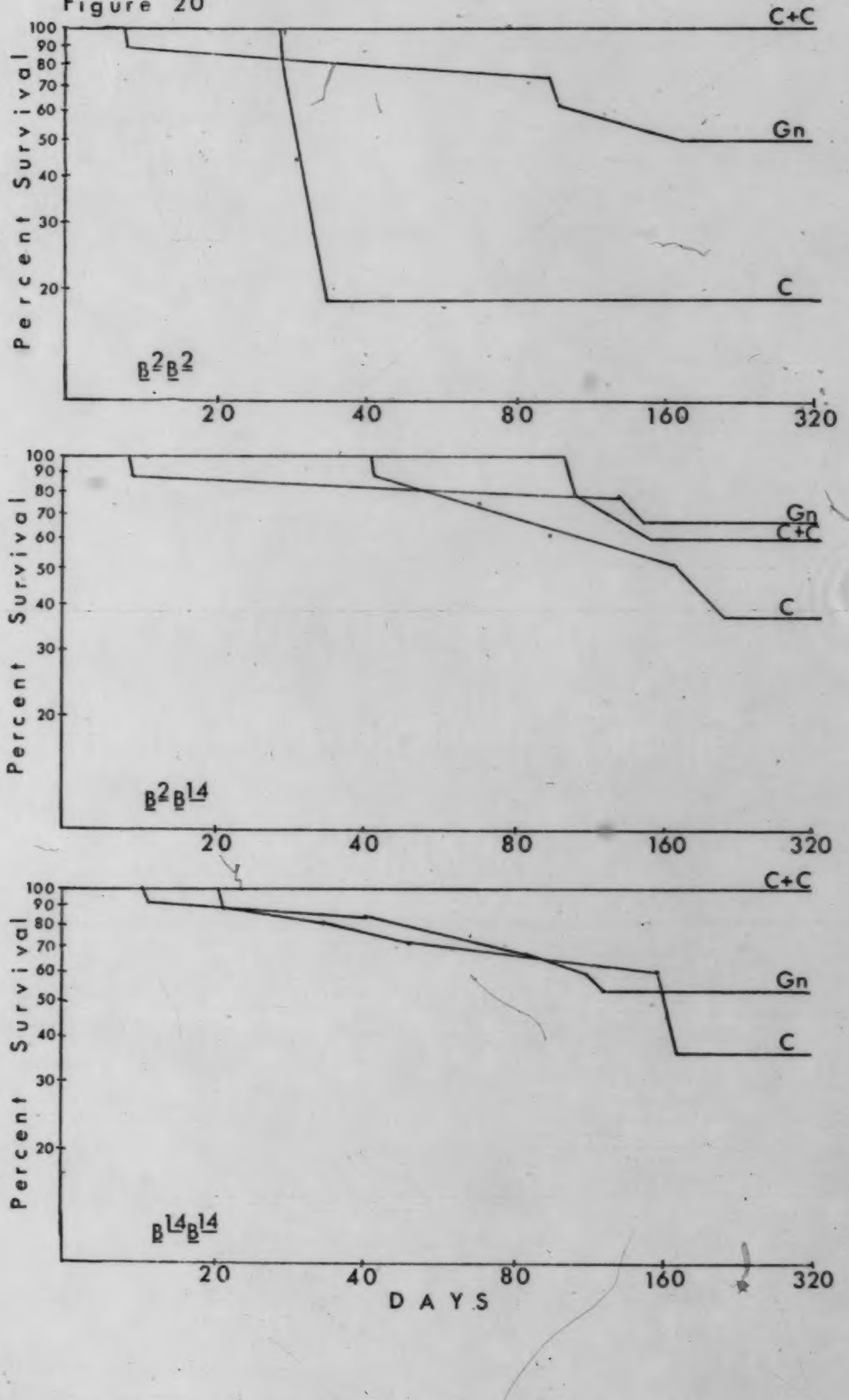

C+C
Gn
C
Percent Survival
100
90
80
70
60
50
40
30
20
B²B²
20
40
80
160
320
Gn
C+C
C
B²B¹⁴
C+C
Gn
C
B¹⁴B¹⁴
DAYS

FIGURE 21: Histogrammatic representation of mean $\log_{10}$ MES with S.E. of embryos injected with cells from thymectomized (T̸) and non-thymectomized (T) gnotobiotic and conventional donors. Embryos represent those differing at the B histocompatibility locus (major B histoincompatibility reaction) and those not differing (minor, non-B, histoincompatibility reaction) pre- and post-conventionalization of the gnotobiotic donors. It should be noted that incubation for measurement of a major B histoincompatibility reaction was four days following injection of the embryos while that for minor (non-B) histoincompatibility reaction was eight days following inoculation. Uninoculated control embryos:

a: B-reaction: 0.8873 pre-conv.
0.8413 post-conv.
b: non-B-reaction: 0.9105 pre-conv.
0.9149 post-conv.

Figure 21

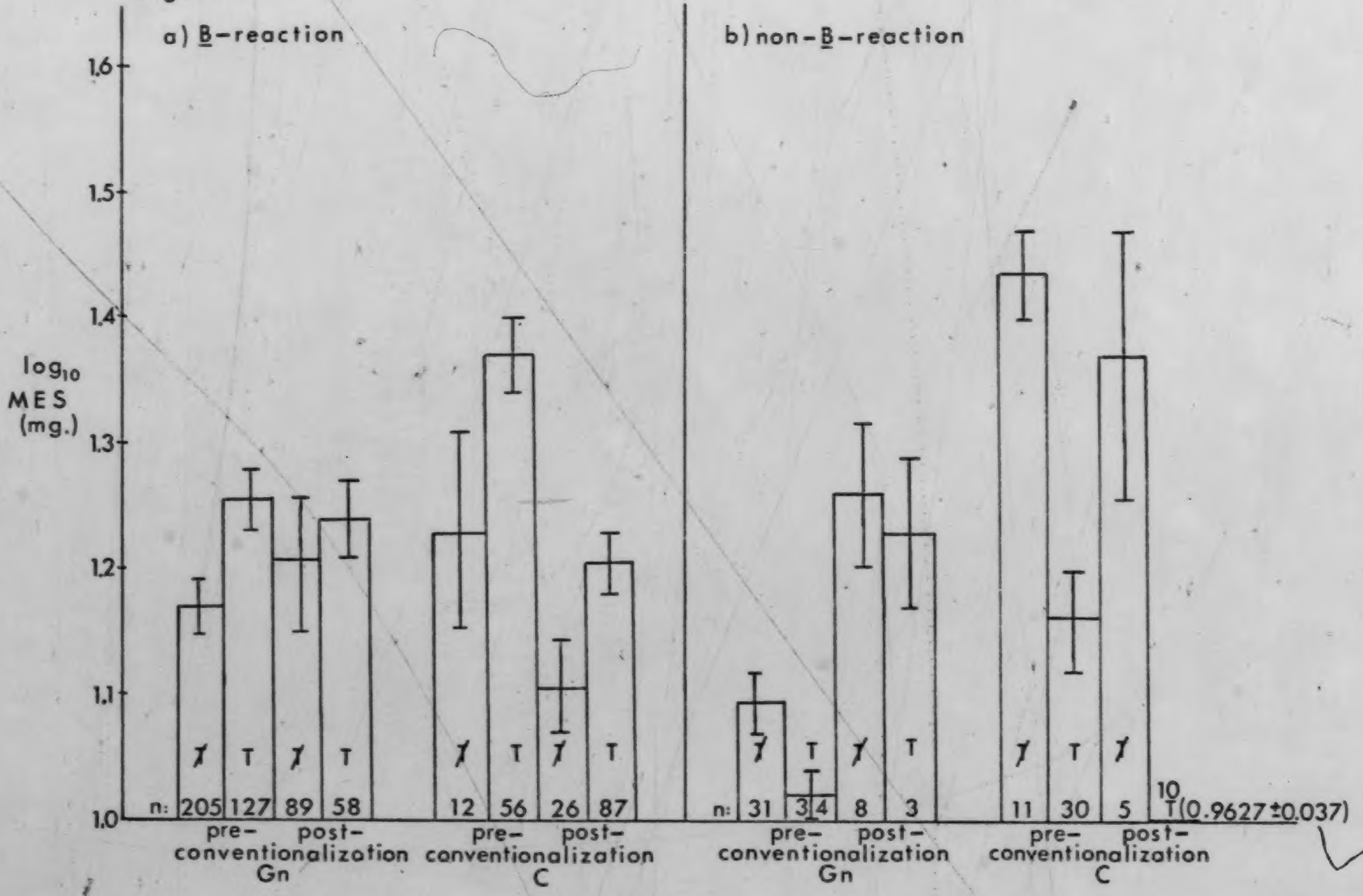

a) B-reaction
b) non-B-reaction
log10 MES (mg.)
1.0
1.1
1.2
1.3
1.4
1.5
1.6
n: 205 127 89 58
12 56 26 87
n: 31 3 4 8 3
11 30 5
T T T T T T T T
pre- post- conventionalization Gn
pre- post- conventionalization C
pre- post- conventionalization Gn
pre- post- conventionalization C
10 T(0.9627±0.037)

FIGURE 22a: Weights of thymectomized ($\bar{T}$) and sham operated (T) $\underline{B}^2\underline{B}^2$ gnotobiotic (Gn) chickens; first hatch.

b: Weights of thymectomized and sham operated $\underline{B}^2\underline{B}^2$ gnotobiotic (Gn) and conventionally housed (C) chickens; second hatch.

Figure 22

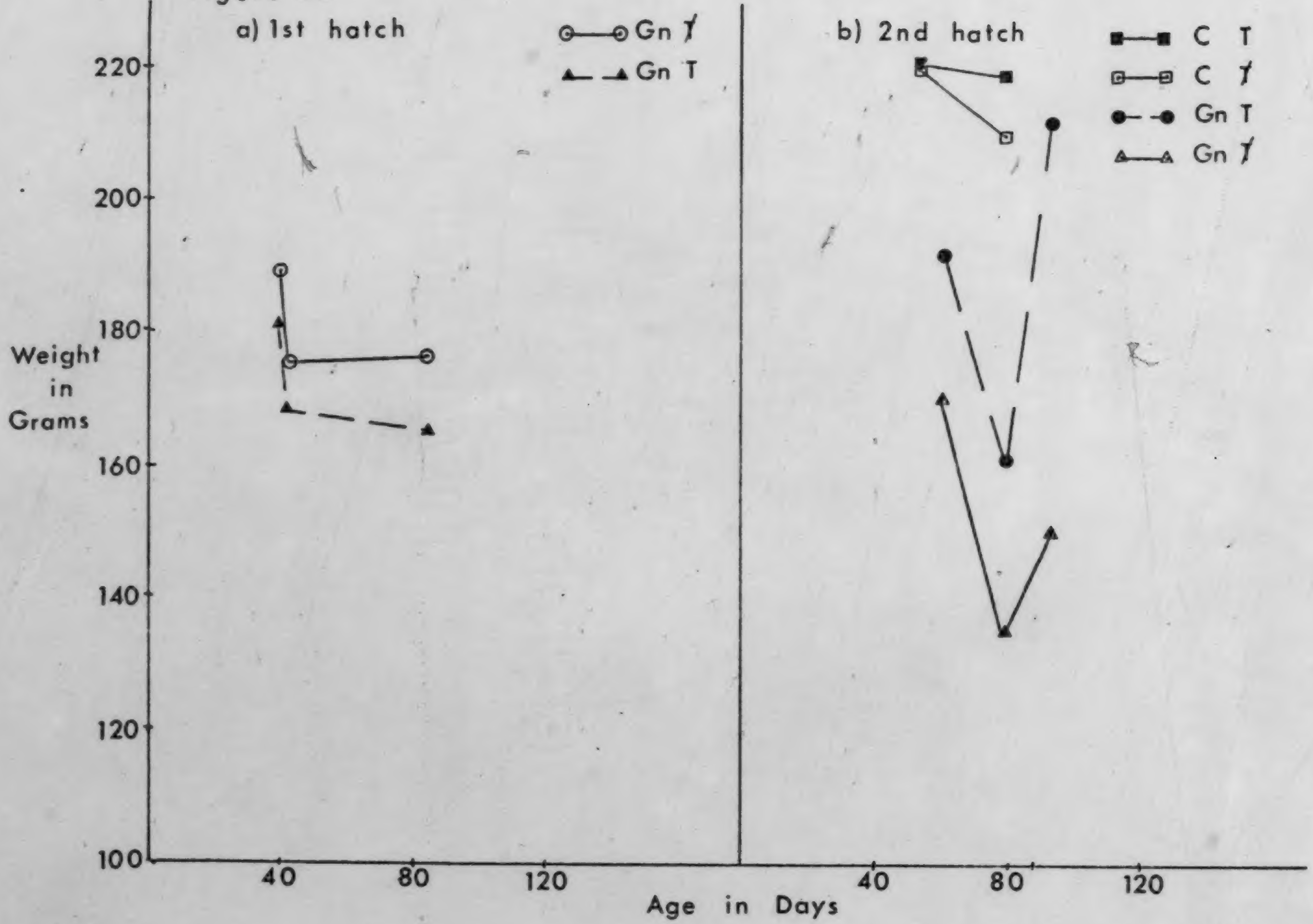
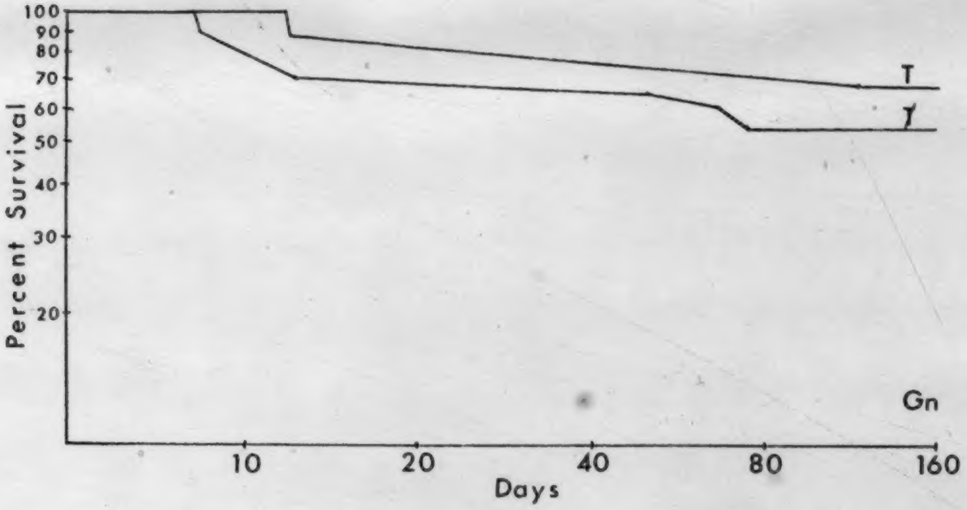

a) 1st hatch
b) 2nd hatch
Gn ♀
Gn T
C T
C ♀
Gn T
Gn ♀
Weight in Grams
220
200
180
160
140
120
100
40
80
120
40
80
120
Age in Days

FIGURE 23: Survival of gnotobiotic (Gn) and conventionally housed (C) thymectomized (T̸) and sham-operated, non-thymectomized (T) chickens.

Figure 23


100
90
80
70
60
50
40
30
20
Percent Survival
T
t
Gn
10
20
40
80
160
Days

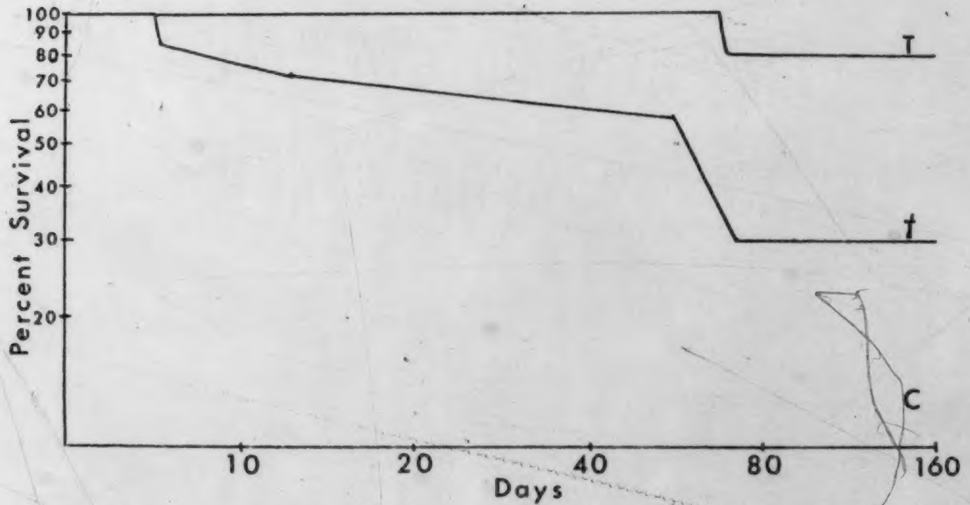

100
90
80
70
60
50
40
30
20
Percent Survival
T
t
C
10
20
40
80
160
Days

FIGURE 24a: Mean $\log_{10}$ CAMP with S.E. of recipient embryos inoculated with cells from surgically bursectomized (B̸), surgically bursectomized plus cyclophosphamide treated (B̸+C), sham-operated (B) and sham-operated plus cyclophosphamide treated (B+C) germfree (GF) and conventional (C) chickens. One totally untreated germfree chicken (U) is included.

b: As in 24a with data grouped according to mean $\log_{10}$ CAMP from each individual donor and the average for these means given for a particular group.

Figure 24

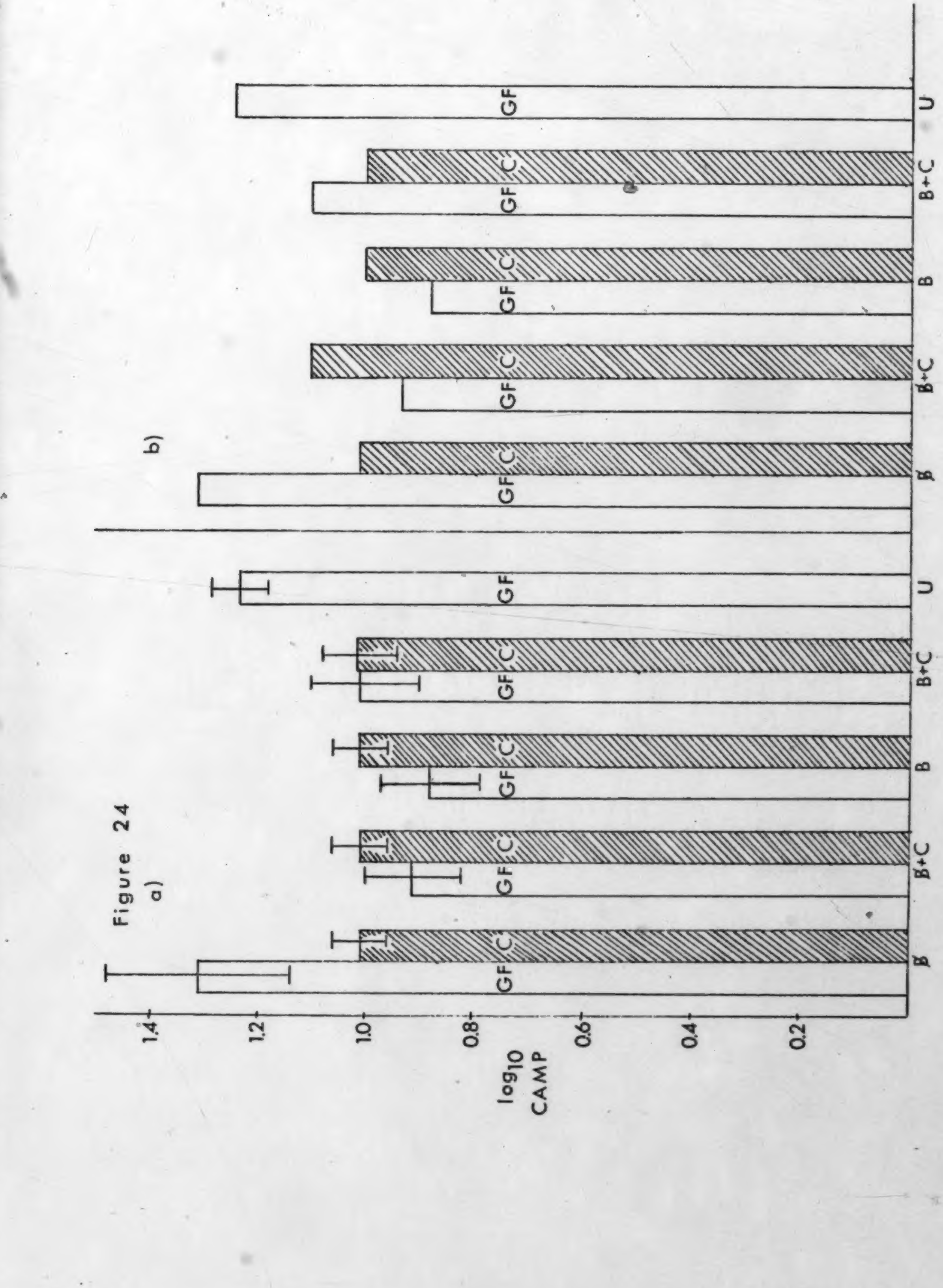


FIGURE 25: Mean $\log_{10}$ CAMP pre-(4 d.) and post-(3 d.) skin testing with mammalian P.P.D. of bursectomized (Ƀ) and sham operated (B) as well as, cyclophosphamide treated (+C) and vaccinated with Mycobacteria in the form of BCG (M).

a: Gnotobiotic animals.

b: Conventionally housed animals.

Figure 25

a) Gn

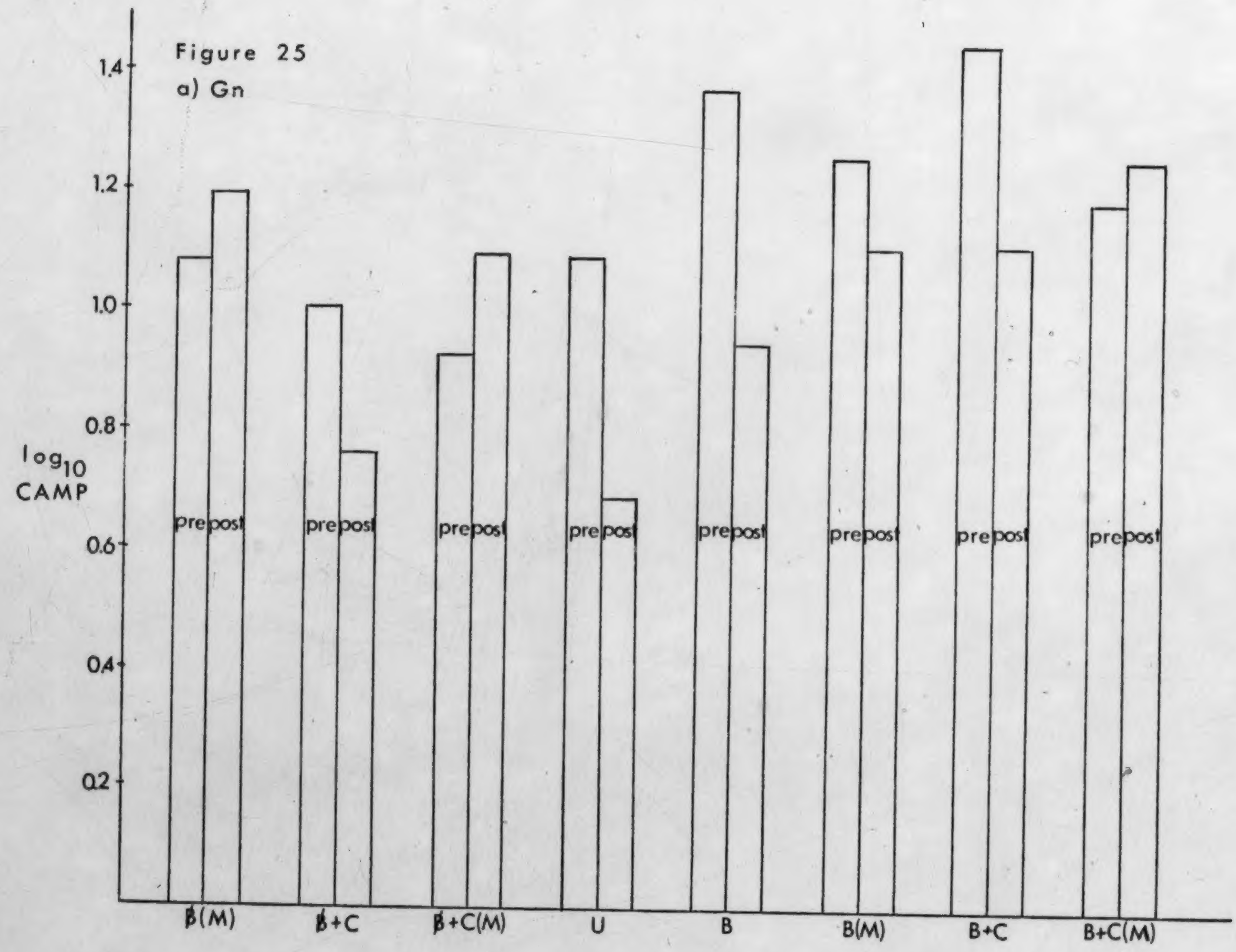


Figure 25
b) C

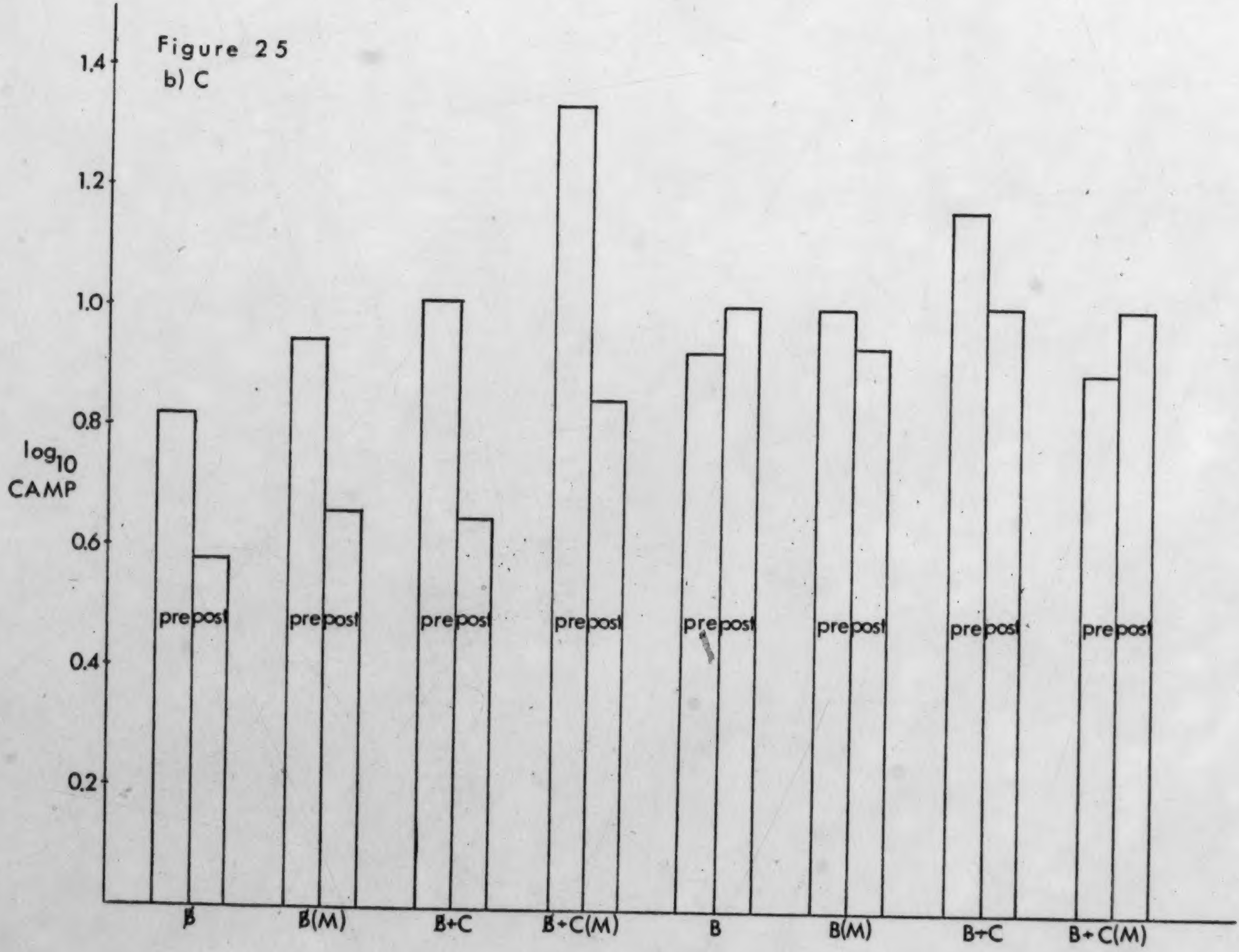


FIGURE 26: Mean $\log_{10}$ CAMP with S.E. pre- and post-skin testing (4 d. pre-, 3 d. post-) with crude BCG. Symbols for treatments of animals as in Figure 25.

a: Gnotobiotic animals.

b: Conventionally housed animals.

Figure 26
a) Gn

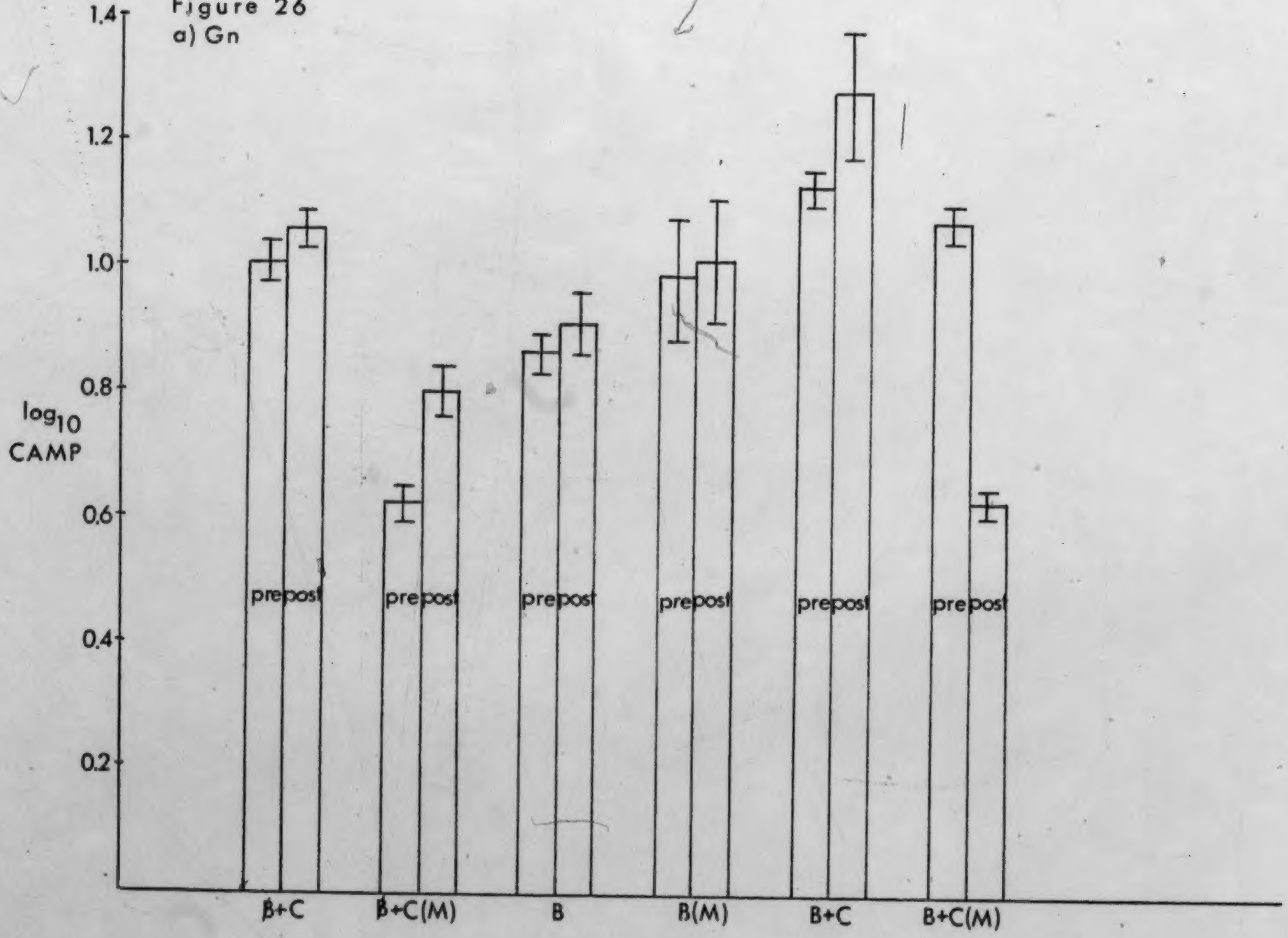


Figure 26

b) C

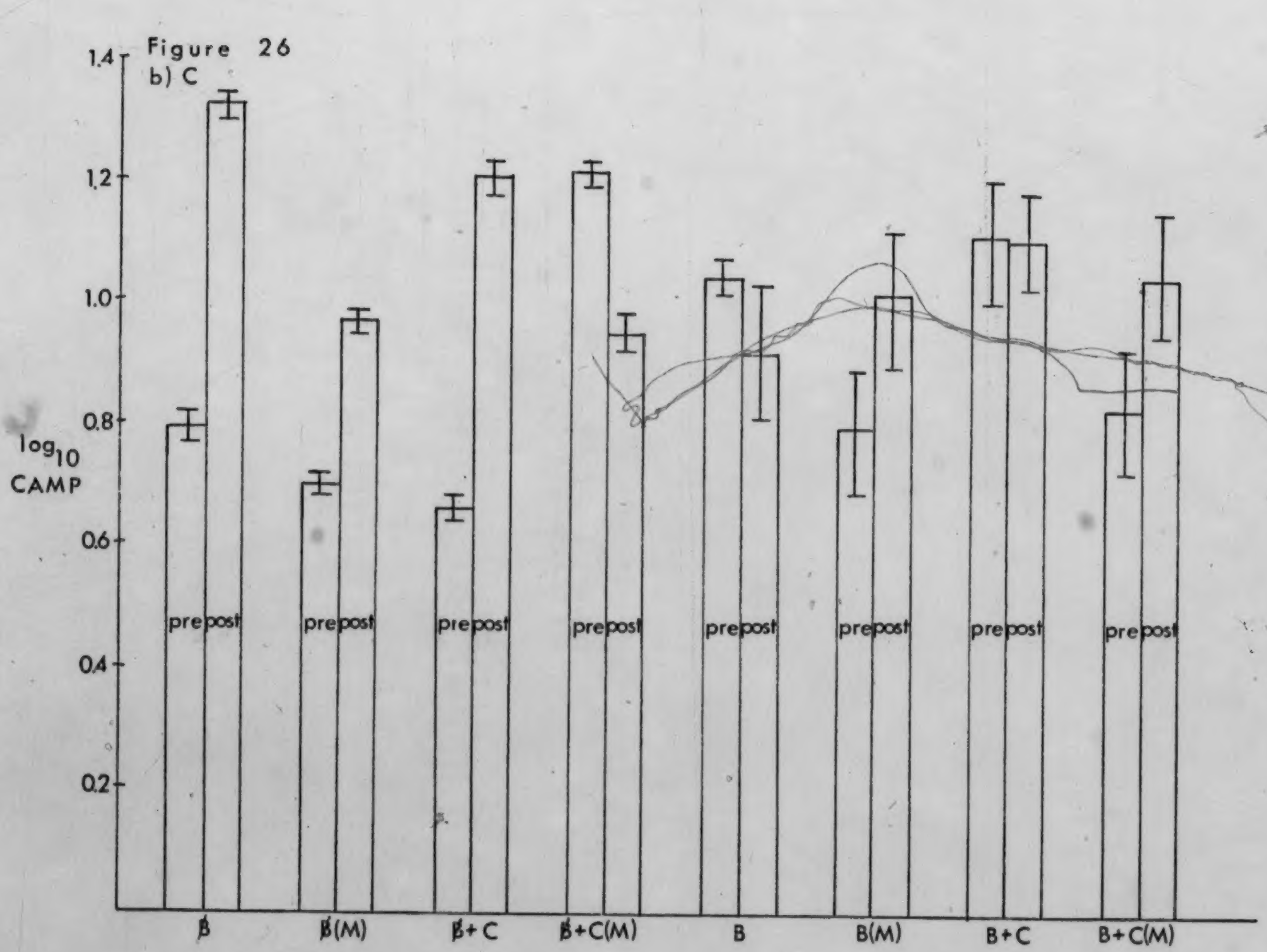


FIGURE 27: Correlation between body weight in grams and spleen weight in milligrams of, (a) gnotobiotic $\underline{B}^{2}\underline{B}^{2}$ bursectomized and sham operated White Leghorns, and (b) conventionally housed $\underline{B}^{2}\underline{B}^{2}$ bursectomized and sham operated White Leghorns.

Coefficient of correlation:

a: Gn: B.W. vs. Spl. W. = 0.7490
df. = 11
highly significant at 1% level.

b: C: B.W. vs. Spl. W. = 0.1715
df. = 15
not significant at 1%.

Ref: Snedecor, p. 174 - table of correlation coefficients at 5% and 1% levels of significance.

Figure 27

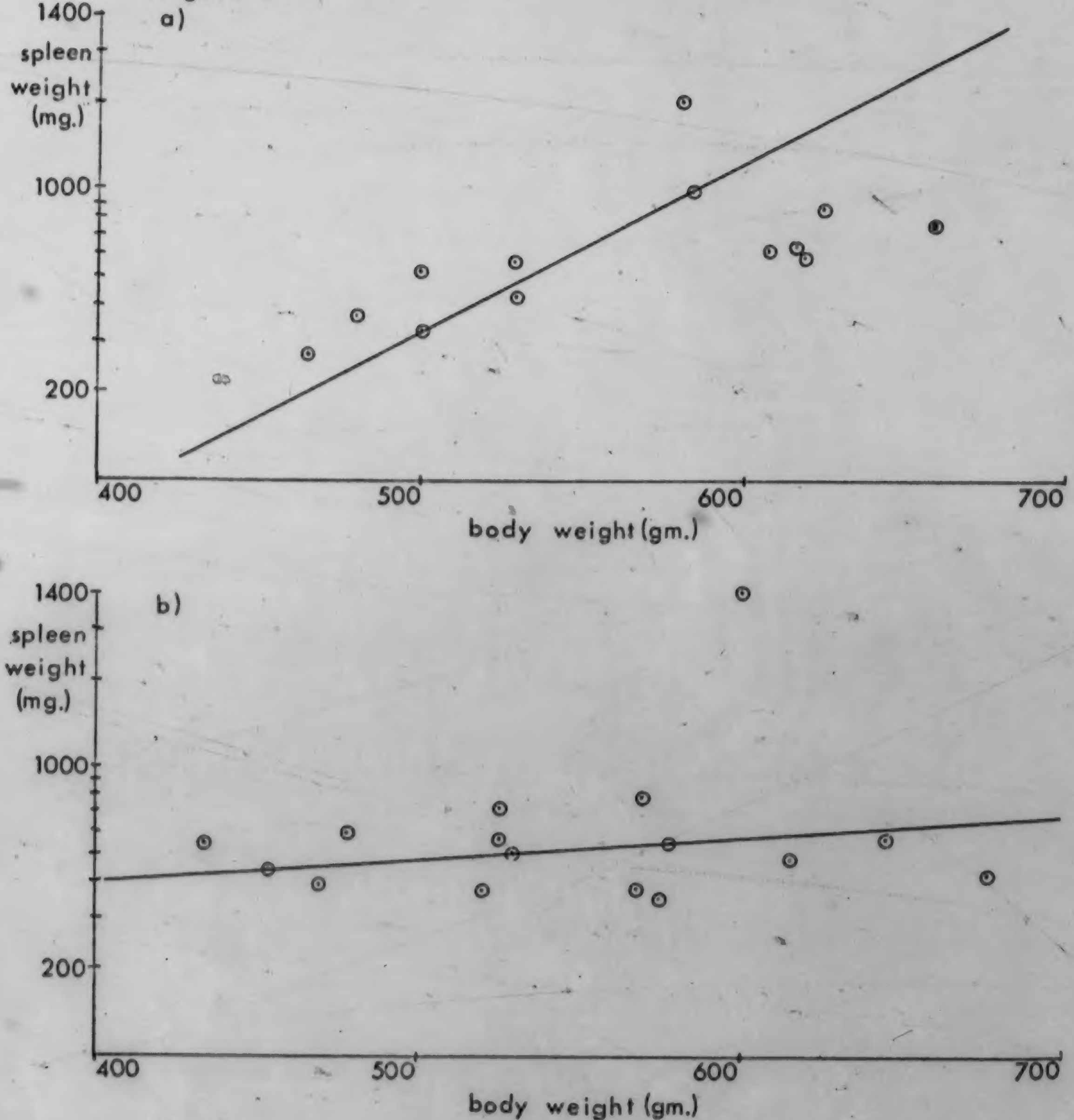
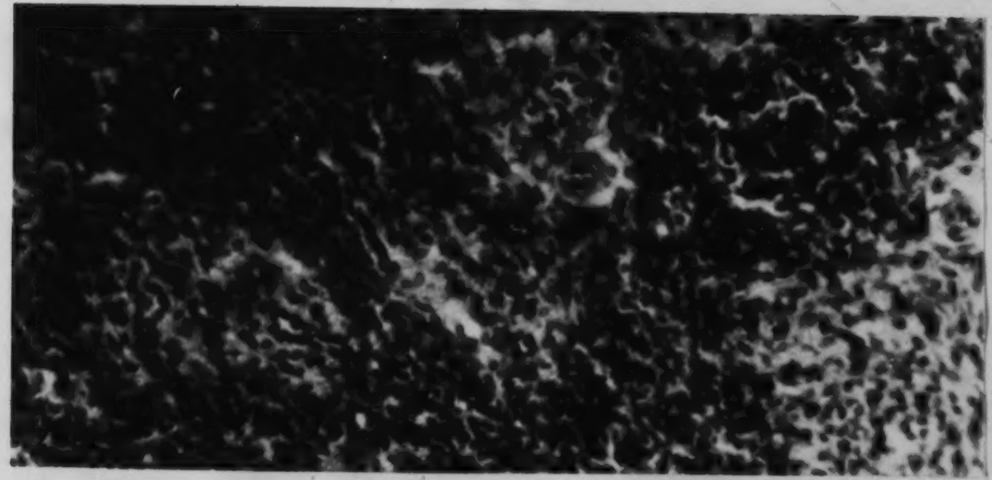

a)
1400
spleen
weight
(mg.)
1000
200
400
500
600
700
body weight (gm.)
b)
1400
spleen
weight
(mg.)
1000
200
400
500
600
700
body weight (gm.)

FIGURE 28a: Representative section from the spleen of bursectomized chickens.

b: Representative section from the spleen of a sham-operated (non-bursectomized) control showing focus of mononuclear cells.



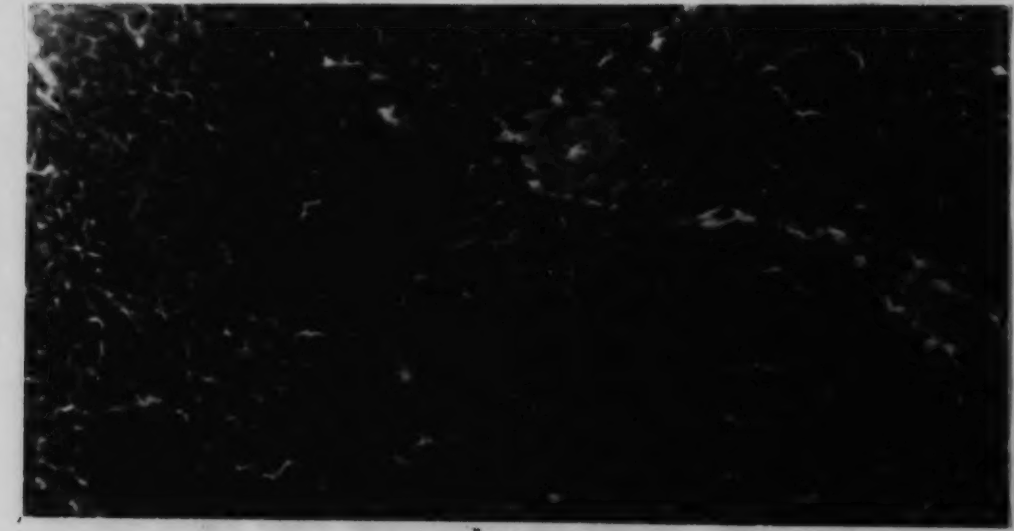


FIGURE 29: Increase in antibody to human A_1 and B, Rh-negative red blood cells pre- and post-injection of SRBC into sham operated gnotobiotic animals.

Note: No bursectomized animals showed any detectable antibody whatever.

FIGURE 30: Increase in antibody to human O (Rh-positive) red cells and SRBC pre- and post-injection of SRBC into sham operated gnotobiotic $\underline{B}^2\underline{B}^2$ leghorns.

Note: No bursectomized animals showed any detectable antibody whatever.

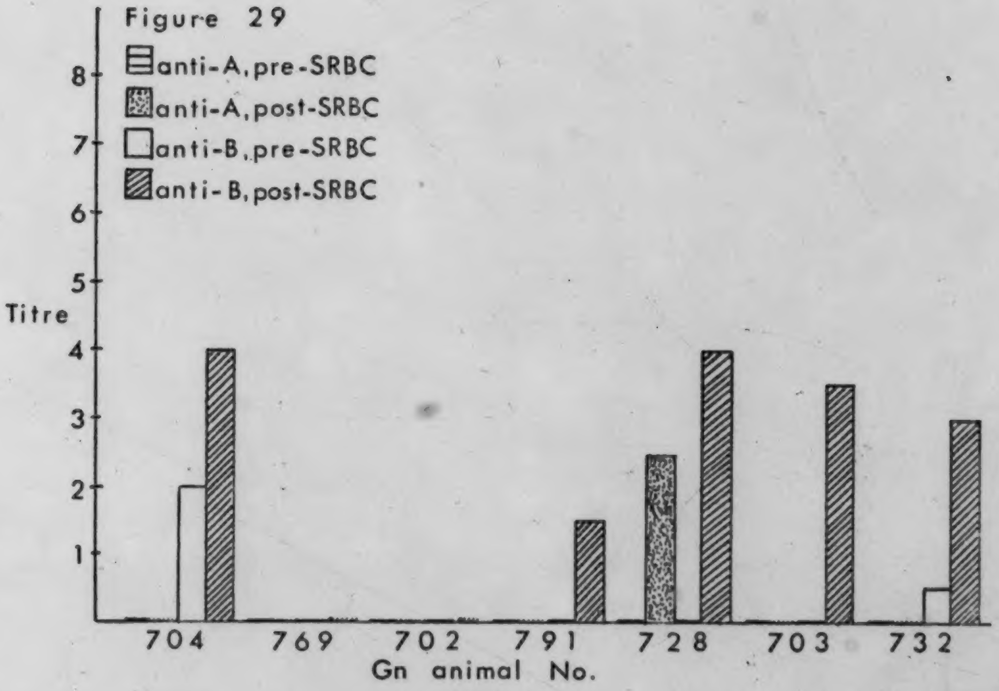


Figure 29

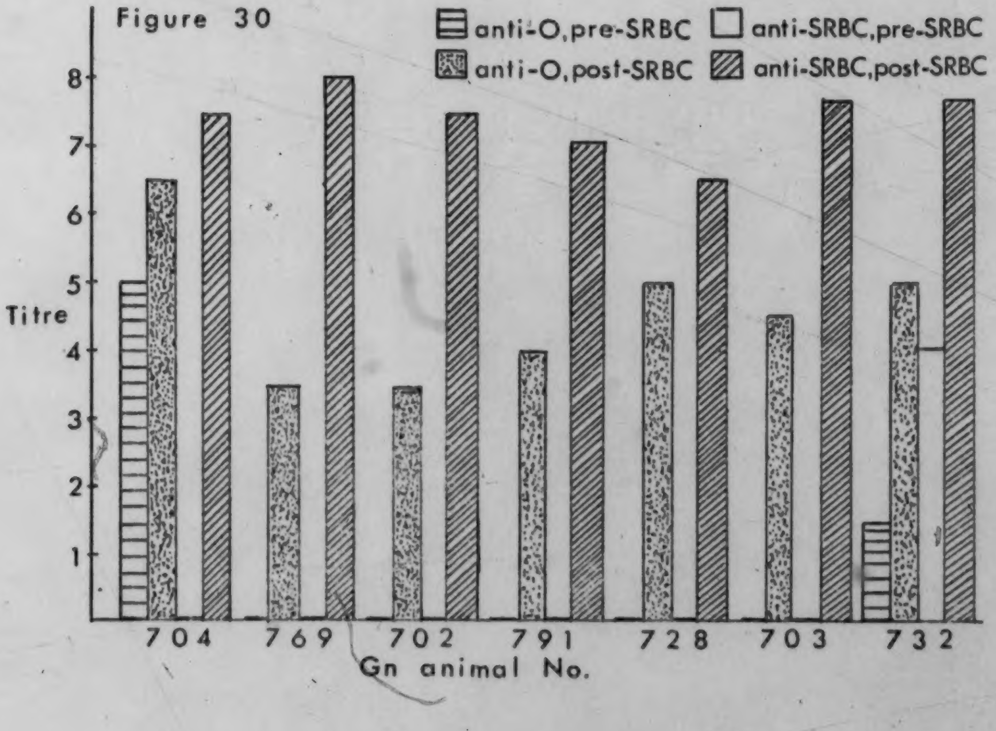


Figure 30

FIGURE 31: Increase in antibody to human A_1 and B, Rh-negative red cells pre- and post-injection of SRBC into sham operated conventional $\underline{B}^2\underline{B}^2$ chickens. Note: No bursectomized animals developed any antibody whatever.

Figure 31

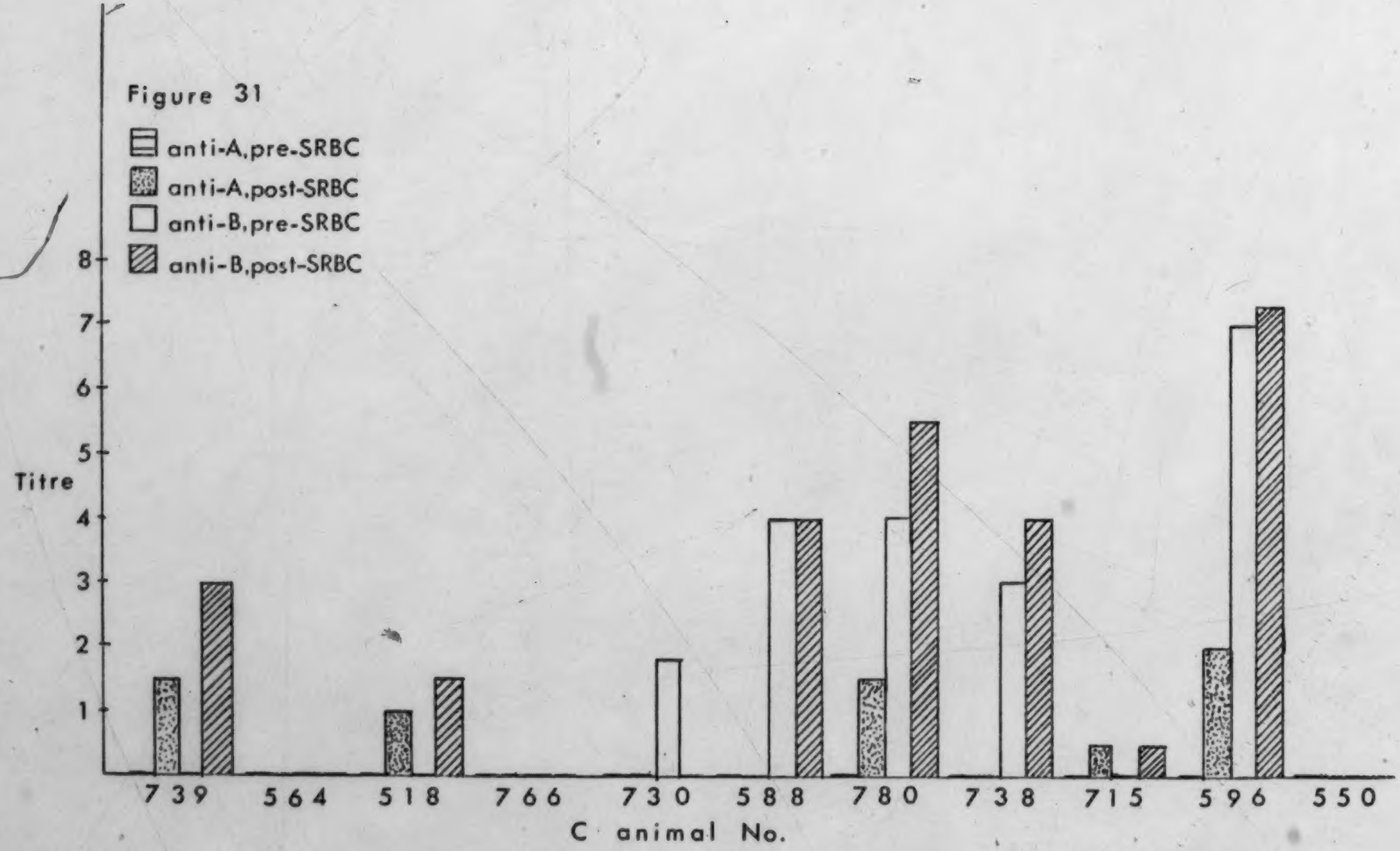
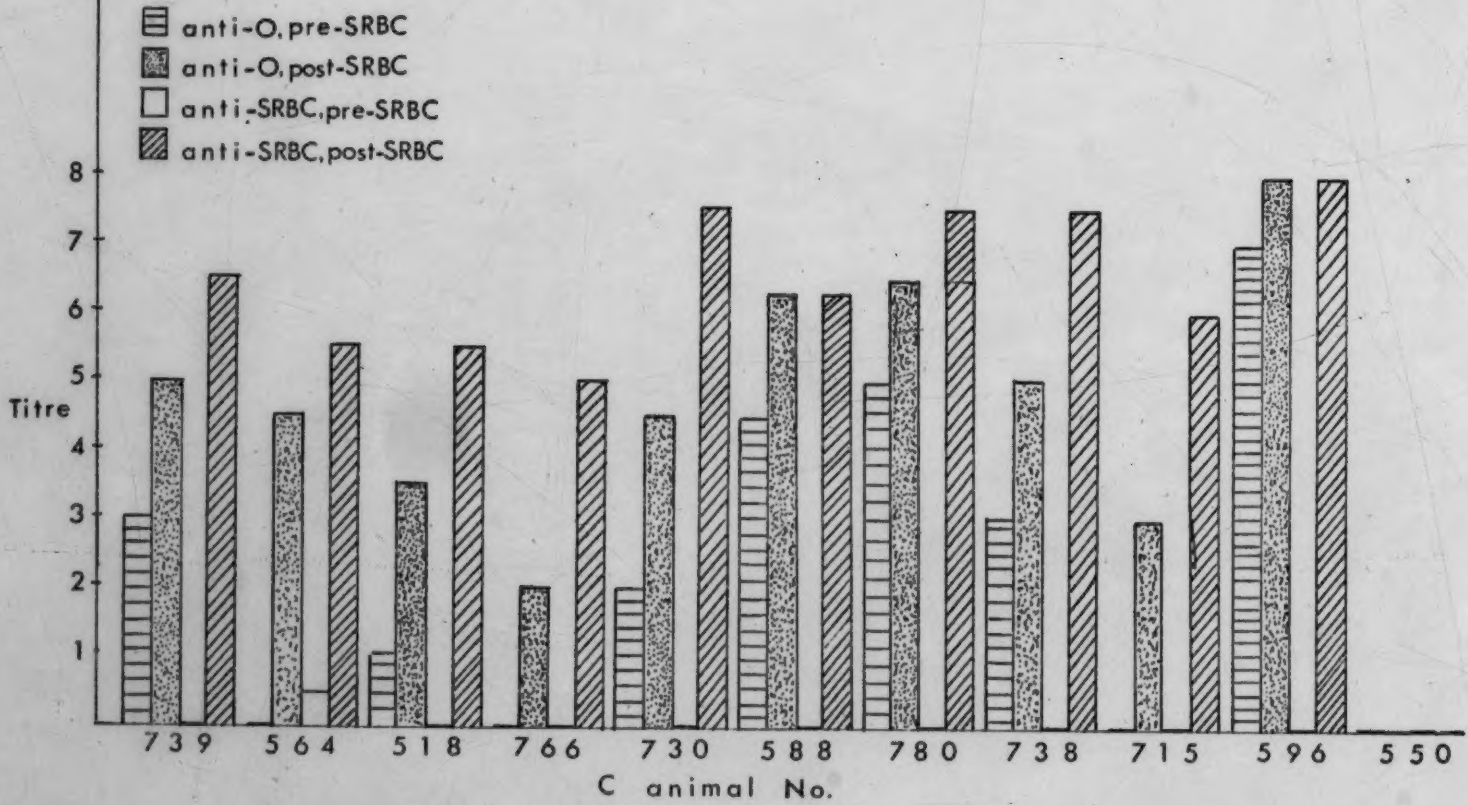

☰ anti-A,pre-SRBC
▩ anti-A,post-SRBC
☐ anti-B,pre-SRBC
▨ anti-B,post-SRBC
Titre
8
7
6
5
4
3
2
1
739
564
518
766
730
588
780
738
715
596
550
C animal No.

FIGURE 32: Increase in antibody to human O, Rh-positive red cells and SRBC pre- and post-injection of SRBC into sham operated conventional $\underline{B}^2\underline{B}^2$ chickens.

Note: No bursectomized animals developed any antibody whatever.

Figure 32



▤ anti-O, pre-SRBC
▩ anti-O, post-SRBC
☐ anti-SRBC, pre-SRBC
▨ anti-SRBC, post-SRBC
Titre
8
7
6
5
4
3
2
1
739
564
518
766
730
588
780
738
715
596
550
C animal No.

FIGURE 33: Survival of bursectomized (B̸) and non-bursectomized (B) $\underline{B}^2\underline{B}^2$ gnotobiotic (Gn) and conventional (C) chickens.

Figure 33

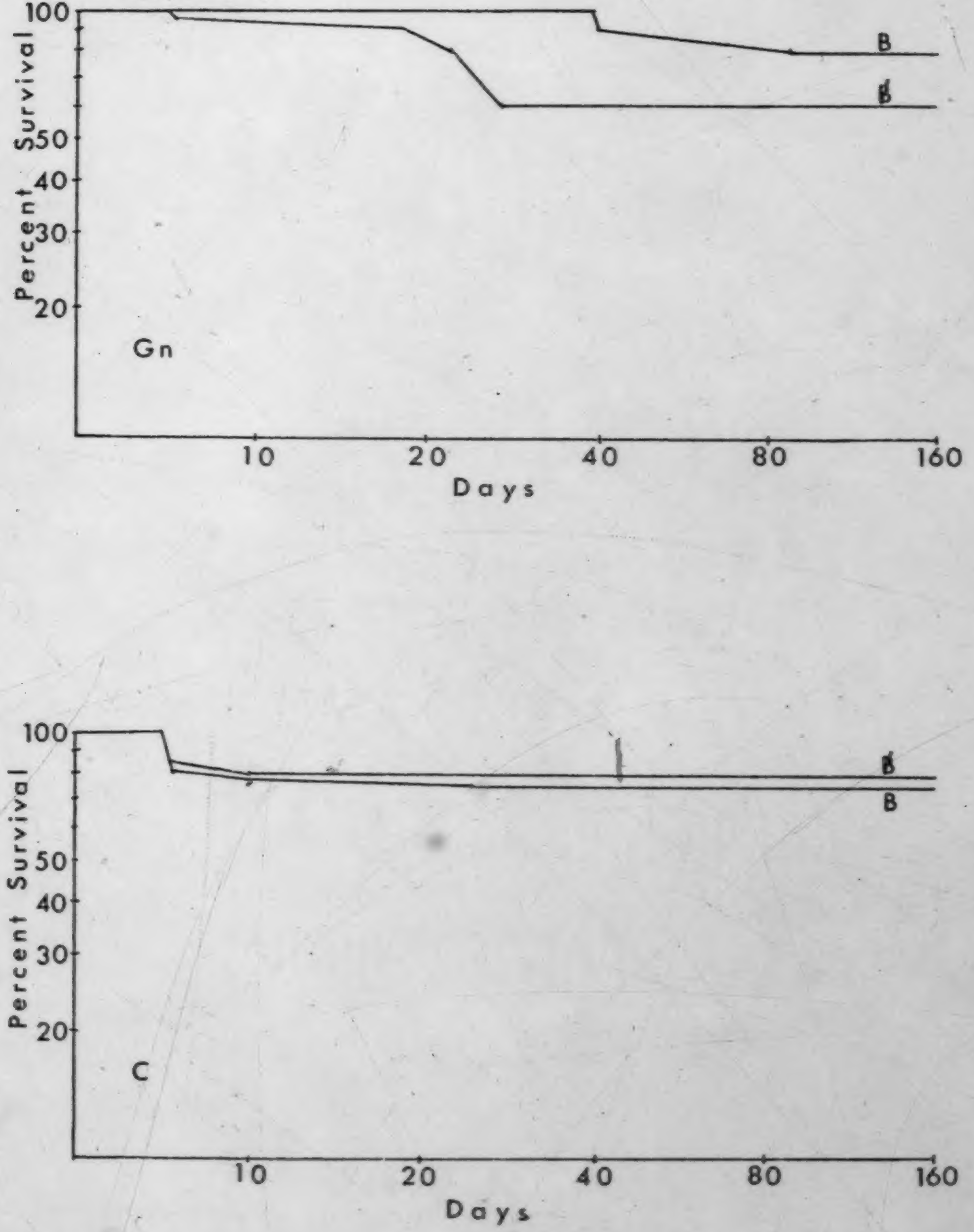

100
50
40
30
20
Percent Survival
B
B
Gn
10
20
40
80
160
Days
100
50
40
30
20
Percent Survival
B
B
C
10
20
40
80
160
Days

FIGURE 34: Acquisition of antibody with age against human A_1 and B, Rh-negative and O, Rh-positive blood cells in germfree English Game Hens.

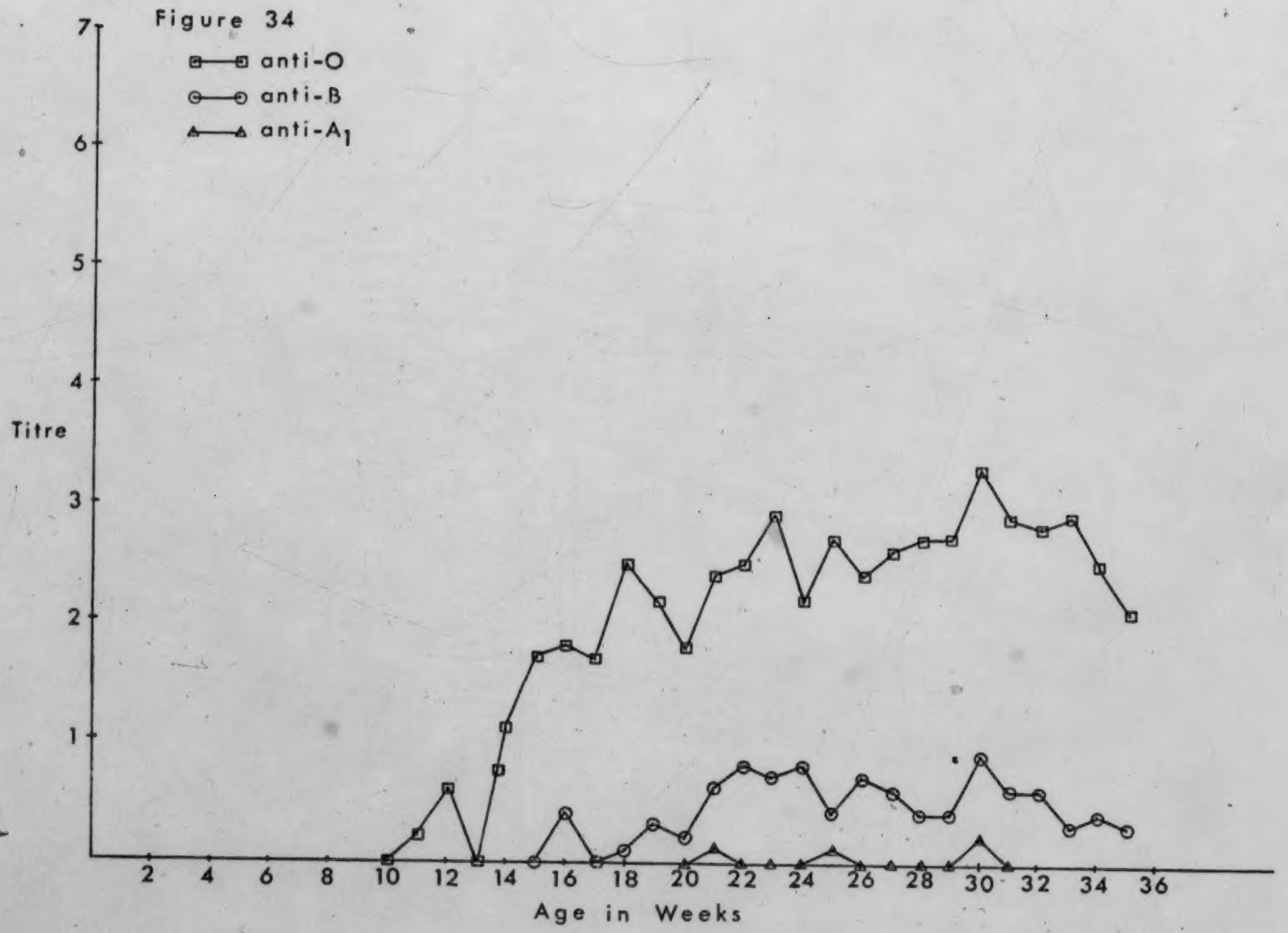


Figure 34

anti-O
anti-B
anti-A$_1$
Titre
1
2
3
4
5
6
7
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
Age in Weeks

APPENDIX

TABLE 1M

MICROORGANISMS IN ISOLATOR OF $\underline{B^2B^2}$ GNOTOBIOTIC CHICKS ON CHICK STARTER DIET FOR FIRST THREE WEEKS AND ON TRYPTOPHANE-DEFICIENT DIET THEREAFTER, EXPERIMENT 1

Animals in Isolator		Organisms* in Isolator:
24018 24017 24016 24015 24014 24013 24012	Hatched July 26/27, 1971	Probable: 1. *Micrococcus* sp. 2. *Staphylococcus epidermidis* 3. *Clostridium perfringens* 4. Gram-negative, non-sporulating aerobic bacillus, not identified.
24019 24020 24021 24027 24028	Hatched Aug. 15, 1971	1. and 2. brought in at 3 to 4 weeks when hatch of August 15, 1971 was brought in. 3. and 4. probably brought in August 4, 1971 with incompletely sterilized feed but recovered one month later (Sept. 13, 1971) in the case of 4. and only on necropsy in the case of 3.

* Tests supportive of identification of the above organisms:

1. Microscopy: Gram-positive cocci in small groups and tetrads:
   Colony morphology: Whitish-gray, non-hemolytic colonies on aerobic blood agar plates (BAP); clear, translucent, small and slow-growing on anaerobic blood agar plates (anBAP) - i.e., preferential aerobic growth.
   Coagulase-negative (-)
   Manitol fermentation-negative (-)
   Catalase-positive (+)
2. Microscopy: Gram-positive cocci in groups.
   Colony morphology: White, opaque, non-hemolytic colonies on BAP, gray on anBAP - preferential growth aerobically.
   Catalase (+)
   Mannitol (-)
   Coagulase (-)
3. Microscopy: Large gram-positive, thick bacilli
   Colony morphology: Gray, shining colonies on an BAP only; double zone of hemolysis.
   Lecithinase (+) - specifically inhibited with *Cl. perfringens* antitoxin on egg plate.
   Glucose (+)
   Mannitol (-)
   Lactose (+)
   Sucrose (+)
   Maltose (+)
   Salicin (-)

TABLE 2M

MICROORGANISMS IN ISOLATORS WITH $B^{\underline{2}}B^{\underline{2}}$, $B^{\underline{2}}B^{\underline{14}}$, $B^{\underline{14}}B^{\underline{14}}$ GENOTYPE GNOTOBIOTIC CHICKS ON CHICK STARTER DIET FOR THREE AND ONE-HALF WEEKS AND ON TRYPTOPHANE-DEFICIENT DIET THEREAFTER EXPERIMENT 2

Isolator #	Chick #	Genotype	Organisms* Isolated
1.	24025	$B^{\underline{2}}B^{\underline{14}}$	Probable:
	24008/12	$B^{\underline{2}}B^{\underline{14}}$	1. _Bacillus_ sp. Probable _Bacillus cereus_
	24010/11	$B^{\underline{2}}B^{\underline{14}}$	
	26527	$B^{\underline{14}}B^{\underline{14}}$	2. _Clostridium perfringens_*
	26547	$B^{\underline{2}}B^{\underline{14}}$	3. _Scopulariopsis_ sp.
	26550	$B^{\underline{2}}B^{\underline{14}}$	Organisms recovered June 18, 1972, approximately one month after hatching.
	26549	$B^{\underline{2}}B^{\underline{14}}$	
	26548	$B^{\underline{2}}B^{\underline{14}}$	
	26851	$B^{\underline{2}}B^{\underline{2}}$	
	26852	$B^{\underline{2}}B^{\underline{2}}$	
	26853	$B^{\underline{2}}B^{\underline{2}}$	
	26854	$B^{\underline{2}}B^{\underline{2}}$	
	26855	$B^{\underline{2}}B^{\underline{2}}$	
2.	26530	$B^{\underline{14}}B^{\underline{14}}$	Probable:
	26529	$B^{\underline{14}}B^{\underline{14}}$	1. _Micrococcus_ sp.*
	26528	$B^{\underline{14}}B^{\underline{14}}$	2. _Staphylococcus epidermidis_*
	26526	$B^{\underline{14}}B^{\underline{14}}$	
	26545	$B^{\underline{2}}B^{\underline{14}}$	3. _Cornyebacterium_ sp.
	26544	$B^{\underline{2}}B^{\underline{14}}$	4. _Clostridium septicum_
	26531	$B^{\underline{14}}B^{\underline{14}}$	Contaminated approximately August 1, 1972.
	26546	$B^{\underline{2}}B^{\underline{14}}$	

* Identification of these organisms carried out in the manner and to the same extent as in Experiment 1, Table 1M.

TABLE 2M (CONTINUED)

Isolator #	Chick #	Genotype	Organisms* Isolated
3.	26537	$B^{14}B^{14}$	Probable: 1. *Bacillus* sp. probable *Bacillus cereus*. 2. *Clostridium perfringens** 3. *Scopulariopsis* sp. Recovered June 28, 1972, approximately 4 weeks after hatching.
	26538	$B^{14}B^{14}$	
	26539	$B^{14}B^{14}$	
	26540	$B^{14}B^{14}$	
	26541	$B^{14}B^{14}$	
	26542	$B^{14}B^{14}$	
	26536	$B^{2}B^{14}$	
	26535	$B^{2}B^{14}$	
	26534	$B^{2}B^{14}$	
	26533	$B^{2}B^{14}$	
Solid	26856	$B^{2}B^{2}$	Probable: 1. *Bacillus* sp. probable *Bacillus cereus* 2. *Scopulariopsis* sp. 3. *Clostridium perfringens** 1. and 2. recovered June 19, 1972. 3. recovered June 23, 1972.
	26857	$B^{2}B^{2}$	
	26858	$B^{2}B^{2}$	

* Identification of these organisms carried out in the manner and to the same extent as in Experiment 1, Table 1M.

Bacillus sp.

Microscopy: Gram-positive, spore-forming bacilli, the width of the bacterial cell being equal to that of the subterminally located spore.
Colony morphology: Large, rough, gray, rhizoid, dry colonies on aerobic BAP, clear spreading growth on anBAP - preferential growth aerobically.
Lecithinase (+) - Probable *Bacillus cereus*.

Corynebacterium sp.

Microscopy: Gram-positive, small, pleomorphic, "false" branching bacilli.
Colony morphology: Small, dry, non-hemolytic colonies on aerobic BAP small, clear, non-hemolytic colonies on anBAP.
Catalase (+).

TABLE 2M (CONTINUED)

Clostridium septicum

Microscopy: Large, gram-positive, spore-forming bacilli, with spores larger in diameter than the bacterial cell located subterminally.

Colony morphology: Large, gray colonies on anBAP only, hemolytic.

Glucose (+)
Mannitol (-)
Lactose (+)
Sucrose (-)
Maltose (+)
Salicin (-)
Lecithinase (-)

Scopulariopsis sp.

Microscopy: Septate mycelium with branched conidiophores and sterigma producing chains of conidia.

Colony morphology: Moderately slow growing colonies on Sabaraud's dextrose agar, white at first, then turning black and powdery with heavy sporulation.

Ref.: Beneke, E.S., 1966. Medical Mycology Laboratory Manual. Burgess Publishing Co., Minneapolis, Minn.

TABLE 3M

MICROORGANISMS IN ISOLATORS OF B^2B^2 THYMECTOMIZED AND SHAM-OPERATED GNOTOBIOTIC LEGHORNS EXPERIMENT 3

Isolator #	Chick #	Treatment	Organisms Isolated and Age of Chicks
1.	29209**	S	Probable: 1. *Cl. perfringens** - 2 weeks 2. *Bacillus* sp.* - 6 weeks 3. *Scopulariopsis* sp.* - 6 weeks
	26873	T	
	29213	T	
	29206	T	
	29205	S	
	29203	T	
	29216**	T	
	26875	S	
	29201	T	
2.	29217	T	Probable: 1. *Cl. perfringens** - 2 weeks 2. *Scopulariopsis* sp.* - 6 weeks
	29206	T	
	29211	T	
	26872	T	
3.	29276***	S	Probable: 1. *Cl. perfringens** - 10 days 2. *Scopulariopsis* sp.* - 6 weeks 3. *Micrococcus* sp.* 4. Gamma *Streptococcus* sp. 5. *Staphylococcus epidermidis** 3., 4. and 5. isolated 2 months after hatching.
	29288/89	S	
	29296/97	S	
	29280/81	T	
	29248***	T	
	29294/95	T	
	29283/83	S	
	29284/85***	T	
	29290/91	T	

* Identification procedures similar to those indicated for Experiments 1, 2, Tables 1M and 2M.

** Animals subsequently moved to Isolator #2.

*** Animals subsequently moved into a fourth (surgical) isolator.

Gamma *Streptococcus* sp.:

Microscopy: Gram-positive cocci in short chains in liquid medium.

Colony morphology: Small gray colonies on aerobic BAP, small translucent on anBAP, no hemolysis, no discoloration.

TABLE 4M

MICROORGANISMS IN ISOLATORS OF B^2B^2 BURSECTOMIZED AND SHAM-OPERATED GNOTOBIOTIC CHICKS EXPERIMENT 4

Isolator #	Chick #	Treatment**	Organisms Isolated and Age of Chicks
1.	704	S	Probable:
	769	S&C	1. _Staphylococcus epidermidis_* - 3 months
	751	B&C	2. Fungus - unidentified - 6 months
2.	791	S&C(M)	Probable:
	702	S(M)	1. _Staphylococcus epidermidis_* - 3 months
	732	U(M)	
3.	703	S&C(M)	Probable:
	763	B&C(M)	1. _Bacillus_ sp.* - 6 weeks 2. _Cl. perfringens_*
S_2	728	S&C(M)	Probable:
	748	B(M)	1. _Staphylococcus epidermidis_* - 3 months
	799	B&C(M)	2. _Mucor_ sp. - 4 months
Solid	773	S&C	Germfree throughout
	722	S&C	

* Procedure for identification of organisms the same as in Experiment 1, Table 1M.

** Legend for Treatment of Animals same as in Table 26.

Mucor sp.

Microscopy: Mycelium aseptate, single and branched sporangiophores, tip of sporangiophore bearing globose sporangium.

Colony morphology: Very rapidly growing filling a Petri dish quickly with aerial mycelium, at first white, then turning brown; many loose black conidia.

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